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Vesicular Stomatitis Virus in Tissue Cultures and Cell Suspensions. (22205)

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The utilization of cultured tissues and cells for the production and assay of viruses has achieved widespread application following the epochal work of Enders, Weller and Robbins (1). The direct use of suspensions of tissue fragments and cells for virus propagation has had, on the other hand, much less appeal even though Frenkel and van Waveren proved the practicability of the method with vaccinia virus nearly 20 years ago(2). More recently, Frenkel employed this method in the production of foot-and-mouth disease virus for use in commercial vaccines(3). A practical test for the determination of virus concentration directly in cell suspensions has been lacking until recently and this appears to have been responsible, in part, for the heretofore limited use of such suspensions in virus studies. Salk *et al.*(4), Lipton and Steigman(5) and Robertson *et al.*(6) have now shown that cell suspensions can be used for the assay of virus and antibodies. In their work poliomyelitis viruses and antibodies have been assayed in replicate suspensions of monkey kidney or Hela cells on the basis that recognizable pH differentials develop between infected and uninfected suspensions due to cell respiration differences.

The present authors have reported that vesicular stomatitis virus (VSV) multiplies and produces cytopathogenic changes in guinea pig kidney and bovine tongue tissues

TABLE I. Yields of Vesicular Stomatitis Virus, New Jersey Type, in Tissue Cultures and Cell Suspensions.

Exp. No.	Description of cultures and suspensions* Method§	Kidney	Vol, ml	Medium	Log TC ID_{50} per ml† No. hr incubation at 37°C 0	24	48	72	96	Virus yield ratio,‡ culture/suspension
1	C	Guinea	2	NF	—	*6.45*	5.07	6.20		.32
	S	pig	2	"	—	4.95	5.45	*6.95*		
2	C	"	2	"	3.70	*6.87*	4.87	3.53	2.00	2.20
	S	"	2	"	3.70	5.20	6.30	*6.53*	6.20	
3	C	"	2	"	2.53	*6.20*	5.70	3.10	2.00	8.53
	S	"	2	"	2.53	3.33	5.20	4.29	*5.33*	
	S	"	200	"	1.53	2.00	2.73	4.33	*5.20*	
4	C	"	2	"	2.53	*6.20*	4.30	2.87	2.33	2.89
	S	"	2	"	2.53	3.53	5.08	*5.53*	5.53	
	S	"	2	"	3.53	3.45	*5.87*	5.53	5.20	
	S	"	200	"	2.53	2.53	4.53	*5.87*	5.20	
	S	"	200	"	3.53	3.33	5.53	*5.70*	5.33	
5 (a)	C	"	2	"	3.47	4.98	*5.30*	4.47		.42
	S	"	200	"	3.47	3.62	4.78	5.15	*5.68*	
(b)	C	"	2	TF	3.47	4.62	*5.70*	4.62		7.95
	S	"	200	"	3.47	4.47	4.68	*4.80*	4.52	
6	S	"	200	"	2.53	2.50	2.83	*4.25*	4.17	
7	S	Bovine	200	"	3.80	4.47	4.93	*5.98*		

* NF and TF, see *Materials and methods.*

† Initial values of TC ID_{50} calculated from titers of inocula and dilutions effected. Italicized values represent maxima.

‡ Maximum TC ID_{50} for culture divided by that of the suspension. In Exp. 3 and 4, TC ID_{50} values employed for suspensions are averages of the maxima.

§ C = Culture; S = Suspension.

grown *in vitro*. It was also observed that this virus inhibited respiration in suspensions of the trypsin-dispersed kidney cells(7). This presumptive evidence for the growth of VSV in cellular suspensions has been confirmed in the present report and data have been obtained on the comparative yields of virus from surviving guinea pig and bovine kidney cells in suspension and from guinea pig kidney following its *in vitro* cultivation.

Materials and methods. Cell preparation. Young guinea pigs weighing approximately 250 g served as a source of kidney tissue. Bovine kidney obtained at an abattoir was cooled to approximately 4°C and brought to the laboratory. The kidneys were cut into small fragments and then digested at 37°C with 0.25% trypsin (1:300 Nutritional Biochemicals Corp.) contained in the phosphate buffered saline (PBS) described by Dulbecco and Vogt(8). After 10 minutes the supernatant fluid of the digest was decanted through 3 layers of gauze. Fresh trypsin solution was added to the undigested residue and the digestion and decantation processes were repeated until most of the kidney tissue was consumed. The combined cellular filtrates were centrifuged at 1,000 rpm for 5 minutes and the sedimented cells were then washed three times in PBS at 600 rpm for 2 minutes. The cells were finally suspended in nutrient fluid, 1 part packed cells to 200 parts of fluid. The nutrient fluid used most extensively consisted of Hanks' balanced salt mixture, horse serum and chick embryo extract, 5:3:2; in Table I and the text, this fluid is referred to as medium NF. A fluid consisting of Hanks' salt mixture, horse serum and hydrolyzed bovine plasma albumin (5% aq. Travamine, Baxter Laboratories), 95:3:2, herein referred to as medium TF was used in some instances. Penicillin G, streptomycin sulfate and phenol red were added to give concentrations of 100 units/ml, 0.1 mg/ml and 0.005%, respectively. *Cell suspensions.* Aliquots of the trypsin-dispersed cells in nutrient fluid were introduced into culture tubes (16 mm dia. x 150 mm) or 1 liter Erlenmeyer

flasks, 2 ml and 200 ml, respectively. After inoculation with virus the cell suspensions were incubated at 37°C. Tubes were held in roller drums rotating at 12 rph and each flask was shaken at one vibration per second in an upright position on a shaking machine having a one-inch stroke. Aliquots of the infected suspensions were assayed in tissue cultures for viral content every 24 hours over a 4-day period. The samples assayed represented pooled 0.05 ml aliquots from each of 10 tubes while those from flasks were single unpooled aliquots. *Tissue cultures for virus production.* Two-ml volumes of the trypsin-dispersed cells in nutrient fluid were introduced into culture tubes. These were slanted and incubated at 37°C. After 4 or 5 days the fluids were changed and at 7 days the cultures contained confluent outgrowths of epithelial cells and fibrocytes. Each of 10 cultures was inoculated with virus and at 24-hour intervals a 0.05 ml sample of infected fluid was removed from each tube for pooling and subsequent virus assay in tissue cultures. *Tissue cultures for virus assay.* These were similar to those used for virus production except that in their preparation 0.5 ml rather than 2.0 ml of the cell suspension was introduced into each tube. The assay cultures were used from 8 to 11 days after preparation. Fluid changes were made at 4 or 5 days and again just before use in titrations. In preparation for the bioassays serial 10-fold dilutions of the infectious fluids to be tested were prepared in PBS. One-tenth ml volumes of the viral dilutions were inoculated into the assay cultures, 5 cultures per dilution. After 48 hours incubation, infection of the cultures was read on the basis of cytopathogenicity and the cumulative 50% infectivity doses for tissue culture (TC ID_{50}) were calculated by the Reed and Muench method(9). *Virus.* Virus-containing fluids for inoculation of cell suspensions and cultures were those obtained after 35 or more consecutive passages of VSV, New Jersey (N. J.) type, in guinea pig kidney cultures. Tissue culture ID_{50} values were determined for all except the first used inoculum.

Results. The data in Table I reveal that the yields of virus from guinea pig kidney cell suspensions (Expts. 1-5) ranged from 4.80 to 6.95 log TC ID_{50}/ml for both media and that the lower limit was 5.20 for NF media only. This compares with a nearly identical range of 5.30 to 6.87 log units for virus produced in guinea pig cultures. A second trial (Expt. 6) with guinea pig cells in suspension in medium TF again resulted in a low yield of virus. On the other hand, bovine kidney cell suspensions in medium TF yielded a relatively high virus titer in Expt. 7. While the virus yield ranges obtained by both methods of production were nearly identical, the yield ratio column of Table I shows that for Expts. 3 and 5b the maximum concentration of VSV in cultures exceeded by approximately 8-fold that found in suspensions. The remaining four comparative experiments showed smaller differences in virus yields by the two methods.

Virus yields in cultures most often reached maximum values within the first 24-hour incubation period and declined precipitously (1.35 to 2.0 log TC ID_{50}/ml) within the next 24 hours. An exception is in Expt. 3 where a sharp drop was not revealed until 48 hours after the maximum yield had been recorded. Expt. 5 differs from the others in that the peak in virus concentration was not reached for 48 hours; the decline thereafter was again rapid.

In cell suspensions, the virus yields are not maximal until 72 or more hours after infection. In contrast to the rapid inactivation of virus which occurs in cultures immediately following peak concentrations, the maximum titers recorded in cell suspensions are not materially changed during the subsequent 24-hour period. Also, a 100-fold increase in suspension volumes did not alter the concentration of the virus in the harvested fluids.

Discussion. It is concluded that guinea pig kidney cultures or cellular suspensions infected with VSV, N. J. type, produce approximately equal quantities of virus when medium NF is employed. The recorded 8.53-fold greater yield of virus in culture than in suspension (Exp. 3) is moderated by the fact that the yields of virus produced in the suspensions of this experiment are at the lower

limit of the yield range for the suspension method when medium NF is employed. Furthermore, the highest yield of virus in the present studies was produced in a cell suspension (Exp. 1), exceeding that from the culture of Exp. 3 by a significant amount. For each of the remaining trials with medium NF the yield ratios do not reveal significant differences in virus production. Three-fold differences in TC ID_{50} values are within the chance range expected in the assay of serial 10-fold dilutions of VSV, 5 cultures per dilution.

In the presence of medium TF, guinea pig kidney cultures appear superior to suspensions for VSV production in the one case studied (Exp. 5b). With this medium more virus was produced in suspension when guinea pig kidney cells were replaced by those from the bovine.

It is considered that the delayed production of virus in suspensions may have been concomitant with the possible initiation of cellular growth. However, repeated counts of nuclei by the method of Sanford *et al.*(10) carried out at 24-hour intervals over 5-day periods on uninfected cell suspensions in flasks failed to reveal any appreciable increases in cell concentrations. Quantitative studies which would relate VSV yields to cell numbers for both trypsin-dispersed kidney cells and their progeny in tissue cultures have not been carried out.

Summary. Vesicular stomatitis virus, New Jersey type, was propagated in suspensions of surviving trypsin-dispersed kidney cells of guinea pigs as well as in cultivated epithelial cells and fibrocytes. Both production methods gave nearly equal virus yields, the highest being approximately $10^{6.9}$ tissue culture ID_{50}/ml. The highest virus concentration in cultures and suspensions were most frequently recorded at 24 and 72 hours following infection, respectively. Cell suspensions were increased in volume from 2 ml to 200 ml without altering the per ml virus yields. After peak virus concentrations had been reached, the rate of destruction of virus infectivity upon further incubation was significantly higher for cultures than for suspensions. Vesicular stomatitis virus was also shown to grow in suspensions of dispersed bovine kidney cells.

1. Enders, J. F., Weller, T. H., and Robbins, F. C., *Science,* 1949, v109, 85; Robbins, F. C., Enders, J. F., and Weller, T. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 370.

2. Frenkel, H. S., and van Waveren, G. M., *Verslag Staatsveeartsenijkundig Onderzoekingsinstitut* 1936-37, The Hague, Netherlands, 1938, p58.

3. Frenkel, H. S., *Am. J. Vet. Res.,* 1951, v12, 187.

4. Salk, J. E., Youngner, J. S., and Ward, E. N., *Am. J. Hyg.,* 1954, v60, 214.

5. Lipton, M. M., and Steigman, A. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 114.

6. Robertson, H. E., Brunner, K. T., and Syverton, J. T., *ibid.,* 1955, v88, 119.

7. Bachrach, H. L., Callis, J. J., and Hess, W. R., *J. Immunol.,* 1955, v75, 186.

8. Dulbecco, R., and Vogt, M., *J. Exp. Med.,* 1954, v99, 167.

9. Reed, L. J., and Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

10. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E., *J. Nat. Cancer Inst.,* 1951, v11, 773.

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Endocrine Factors in Pathogenesis of Experimental Poliomyelitis in Hamsters.* Role of Inoculatory and Environmental Stress. (22206)

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Golden hamsters are susceptible to intracerebral but refractory to intraperitoneal inoculation of MEF_1 virus(1,3). Susceptibility of intracerebrally inoculated animals undergo significant alterations as a result of natural or experimental disturbances in the adrenal-testes endocrine equilibrium(2). This paper reports the effect of interacting stress, endocrine function, and route of inoculation upon poliomyelitis susceptibility in hamsters.

Materials and methods. Male Golden hamsters 25-30 g were inoculated with MEF_1 virus or control sterile mouse brain emulsion (SMBE) in equal volume. Animals were killed in groups of 20, daily for 5 days, then at 7, 10, 12, 15, and 28 days following inoculation. Weights of adrenal glands, testes, thymus, and interscapular brown fat (ISB) of animals inoculated with virus were compared with those inoculated with the SMBE and with uninoculated controls sacrificed the same days. The following volumes of MEF_1 virus suspension, diluted 1/100 in saline, were used: 0.05 ml intracerebrally, 0.02 ml intraspinally, 0.5 ml intraperitoneally, and 0.2 ml intramusculary and subcutaneously. Paralytic incidence and mortality were noted daily for 21 days. Animals dying without paralytic symptoms have been excluded. Histological examination of the spinal cord, and mouse inoculation were periodically used to control clinical observations. The experiments were repeated a number of times and the results were averaged per group, per season, and per year. Except for the investigation of climatic stress, all animals were kept in the constant environment of air conditioned rooms at approximately 23°C. The results were analyzed by statistical procedures previously described(2). The association between series, *i.e.*, proportion

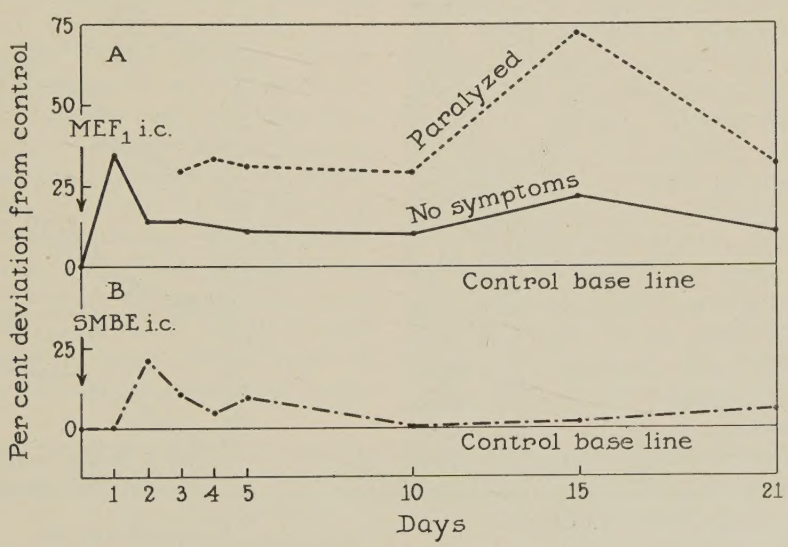

FIG. 1. Percent deviation from control of mean adrenal weight following intracerebral injection in Golden hamsters: A. in 206 animals injected with MEF_1 virus; B. in 625 animals injected with sterile emulsion (SMBE). Base line represents non-injected controls sacrificed the same time and in equal number as experimental animals.

* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Effect of Preparatory Pathogen-Free Intracerebral Injection (SMBE) upon Susceptibility of Golden Hamsters to Poliomyelitis Virus (MEF_1) Inoculated Intraperitoneally.

Treatment		No. animals	Incidence of paralysis, %	Statistical significance "P"
Intracerebral	Intraperitoneal			
Sterile M.B.E.* .05 cc	MEF_1 virus 5 × .5 cc	125	16.00	.0001
None	*Idem*	254	3.93	

* M.B.E. = Mouse brain emulsion.

of poliomyelitis incidence (Y axis) and proportion of hypertrophic adrenals (X axis), under varying stressing conditions, was analyzed by using the correlation technic(18,19).

Results. Effects of inoculatory trauma. The effect of intracerebral inoculation upon the endocrine glands was studied on 1500 hamsters. Animals inoculated with virus intracerebrally averaged significant adrenal hypertrophy within a 5-day period following injection (Fig. 1). Alterations of the average adrenal weight noted beyond this period were statistically insignificant. Average weights of thymus, testes, and ISB showed no significant changes within the initial 5-day period. Endocrine alterations of the same pattern were found when SMBE was substituted for the viral inoculum (Fig. 1), indicating that the observed adrenal hypertrophy was produced by the intracerebral inoculation, rather than by MEF_1 virus. Among animals inoculated with virus, adrenal hypertrophy was found in the paralyzed animals regardless of elapsed time between intracerebral inoculation and start of paralysis (Fig. 1). To determine whether the refractory state to intraperitoneal inoculation virus could be eliminated by a preliminary sterile intracerebral injection, animals were injected intracerebrally with SMBE, after which MEF_1 virus was given intraperitoneally every other day, 3 or 5 times. Poliomyelitis, confirmed clinically and pathologically, developed in 12-25% of the treated animals. Control groups, repeatedly inoculated with virus intraperitoneally, only developed the disease in 2-6% (Table I).

Persistence of MEF_1 virus in various inoculated tissues. A single intraperitoneal inoculation of virus given the same day as the sterile intracerebral injection did not produce poliomyelitis. This suggests that rapid disappearance of the virus precluded dissemination before initiation of the endocrine alterations which usually appear 48 hours after the intracerebral trauma (Fig. 1). To determine whether there is any significant difference in persistence of poliomyelitis virus within the cranial and peritoneal cavities, several groups of infected animals were given a single injection of 5 mg cortisone at different intervals following virus inoculation. Cortisone produced

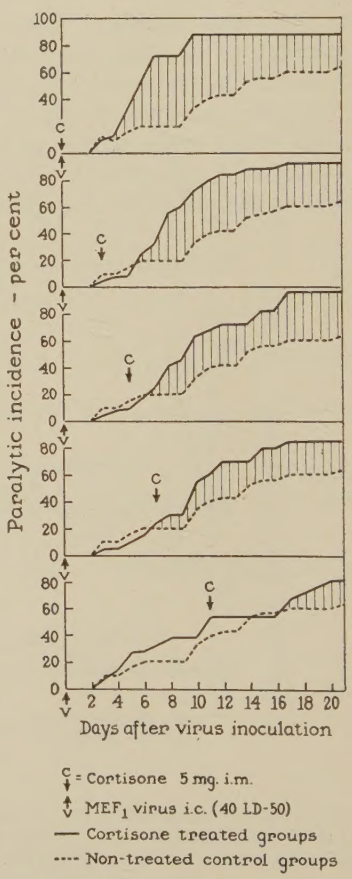

FIG. 2. Conversion of quiescent poliomyelitis into paralytic disease with the aid of cortisone given at varying intervals after intracerebral inoculation of virus.

TABLE II. Frequency of Hypertrophic Adrenals According to Age in Populations of Normal Hamsters Kept in Constant Environment of An Air Conditioned Laboratory.

Body wt, g	No. animals	Proportion of adrenals over 14 mg/100 g, %
25–35	403	17.61
35–50	538	19.70
50	135	46.66

enhancement of paralytic incidence as late as 11 days following intracerebral inoculation, indicating that potent virus was still present in a quiescent state. Conversely, no paralysis or death was produced by cortisone beyond the third day following intraperitoneal, intramuscular or subcutaneous inoculation of virus thereby suggesting that the virus was rapidly destroyed when introduced peripherally, unless endocrine alterations like those induced by a sterile intracerebral injection favor its progression and dissemination.

Endocrine reactivity to stress and susceptibility to poliomyelitis virus. Assuming that individual or seasonal variations in susceptibility to poliomyelitis may be associated with individual or seasonal deviations from the general pattern of endocrine reactivity to inoculatory trauma, adrenal reactivity was investigated and compared with susceptibility to poliomyelitis virus in 5000 hamsters maintained in the relatively constant environment of an air-conditioned laboratory. The weight of 14 mg per 100 g body was considered as the boundary limit between normal and hypertrophic adrenals. This represents the median figure between the mean value found in 299 hamsters submitted to normal climatic fluctuations and the mean adrenal weight of 486 animals kept in air-conditioned rooms and sacrificed during the same seasonal periods as the former. Actually, more than 80% of 941 young hamsters (25-50 g), kept in a constant environment and autopsied at various periods of 1954, showed adrenals weighing less than 14 mg/100 g. The frequency of adrenal hypertrophy increased with the age, due possibly to repeated occasional stresses associated with daily care, *i.e.*, feeding, cleaning, etc. (Table II).

When SMBE was injected intracerebrally in young hamsters, 33% of 327 animals sacrificed during the following 5 days, showed adrenal hypertrophy. When poliomyelitis virus was similarly injected in 372 animals, 35% developed paralytic symptoms during the first week of observation. Table III shows that seasonal variations observed in adrenal reactivity to inoculatory trauma were paralleled by similar fluctuations in the paralytic incidence of the first week following inoculation.

As may be seen from Table III, the paralytic incidence of the first week following intracerebral inoculation of virus, represents only 2/3 of the total incidence recorded during a standard observation period of 21 days under constant environmental conditions. Based upon the assumption that the additional 1/3 paralytic incidence may be incidental to additional stresses, such as daily examination of the animals, 100 hamsters were inoculated with MEF_1 virus intracerebrally then separated into 2 groups. One group was examined routinely every day, while the other group was kept undisturbed for 21 days. Table IV shows that both morbidity and mortality were higher in the group examined daily, than in the non-disturbed group.

TABLE III. Seasonal Fluctuations in Adrenal Reactivity to Inoculatory Trauma as Compared with Parallel Variations in Susceptibility to Poliomyelitis Virus.

	Intracer. inj. S.M.B.E.*		Intracer. inj. of MEF_1 virus		
Seasonal period	No. animals	Incidence of adrenal hypertrophy, %	No. animals	Incidence of paralysis 1st wk, %	Total paralytic incidence 3 wk, %
5/54	99	31	89	38	61
9/	129	24	147	26	40
2/55	99	46	136	42	66
Yearly avg	327	33	372	35	55

* S.M.B.E. = Sterile mouse brain emulsion.

TABLE IV. Effect of Daily Examination upon Paralytic Incidence and upon Mortality from Poliomyelitis in Golden Hamsters Inoculated with MEF_1 Virus Intracerebrally, 50 Animals in Each Group.

Groups	Paralytic incidence, %	Mortality, %
Examined daily	64	44
Not disturbed	52	30

Though the difference was found statistically insignificant, it persisted when the experiment was repeated on 175 additional animals.

The role of environmental stress in enhancing paralytic incidence in intracerebrally inoculated hamsters is demonstrated in Table V. The paralytic incidence is significantly higher in animals kept in the variable environment of normally heated rooms than in animals kept in air-conditioned laboratories. A significant increase in belated paralytic incidence, *i.e.*, appearing beyond the first week following inoculation, seems to be the main effect of the stressing environment. This explains the paradoxical observation that, despite the greater morbidity, the mean incubation period was longer in a varying than in a constant environment due to the number of belated paralytic cases provoked by environmental stress (Table V).

Groups of animals maintained in refrigerated rooms at 5°C or dark rooms at 23°C showed adrenal hypertrophy in a percentage similar to the percentage of paralytic poliomyelitis observed under similar environmental conditions (Table VI).

Statistical analysis of the association between series in 7 pairs of groups totaling 1602 animals submitted to varying experimental stresses as shown in Tables III and VI, revealed a linear correlation of high statistical significance between the percentage of adrenal hypertrophy in one series and the percentage of paralytic poliomyelitis in the other. As a corollary observation, 82% of the animals developing poliomyelitis showed adrenals over 14 mg/100 g, at the time when the paralytic symptoms first became evident.

Comparative effects of various inoculation procedures. In hamsters kept in an air-conditioned environment, a sterile intraspinal in-

TABLE V. Progression of Paralytic Incidence following Intracerebral Inoculation of MEF_1 Virus in Groups of Hamsters Kept in Variable Environment as Compared to Groups Kept in Air Conditioned Rooms at 23°C.

Environment	No. animals	Early paralysis 1st wk, %	Significance "P" of difference	Late paralysis 2nd & 3rd wk, %	Significance "P" of difference	Total paralysis 3 wk, %	Significance "P" of difference	Mean incubation period, days
Variable (normally heated room)	600	26.16		35.50		61.66		9.40
Constant (air conditioned)	1333	33.53	.001	19.05	Highly significant*	52.58	.001	7.53

*.0000000001.

TABLE VI. Incidence of Adrenal Hypertrophy as Compared to Incidence of Paralytic Poliomyelitis in Groups of Hamsters Submitted to Various Environmental and Dietary Conditions for 3 Weeks.

Treatment	Adrenal reactivity			Susceptibility to MEF_1 intracer.	
	No. animals	Avg adrenal wt, mg/100 g	Incidence of adrenal hypertrophy, %	Total No. animals	Paralytic incidence, %
Refrigeration	139	15.14	61.39	86	65.11
Dark room	183	15.07	52.33	93	58.06
Protein enriched diet	125	14.17	54.40	132	59.84
Idem + S.M.B.E., intracer.*	144	14.76	52.77		

* Intracer. inoculation of .05 cc sterile mouse brain emulsion.

jection was followed by a larger proportion of adrenal reactions than an intracerebral one, *i.e.*, 46% as compared to 19% during April 1955. The incidence of paralytic poliomyelitis also was significantly higher in intraspinally than in intracerebrally inoculated animals. The mean incubation period was shorter after intraspinal than after intracerebral inoculation of virus (Table VII). An intraspinal injection with SMBE prior to and on the same day as the intracerebral inoculation of MEF_1 virus, raised the incidence of paralysis to the same level as in animals receiving virus intraspinally. The mean incubation period remained however, within the range of intracerebrally inoculated animals. A sterile intracerebral injection preceding administration of virus intraspinally did not change significantly the paralytic incidence or the incubation period as compared to controls inoculated only intraspinally (Table VII).

Adaptation to stress and susceptibility to poliomyelitis virus. Intracerebral inoculation of MEF_1 virus after varying periods spent in cold environment, resulted in a decreased incidence of poliomyelitis in proportion to the time spent in the cold environment prior to inoculation, *i.e.*, 70% when refrigeration started on the day of inoculation, 66% in groups refrigerated for 5 days prior to inoculation, and 57% in groups refrigerated for 10 days preceding the inoculation. Chronic adrenal hypertrophy was found in 86% of hamsters kept under protracted climatic stress. When poliomyelitis virus was injected intracerebrally in similar groups, paralytic incidence was only 66%, suggesting that, with time, adaptation had compensated for the enhancing effect of the adrenal hyperfunction in about 20% of these continuously stressed animals (Table VIII). Conversely, spontaneous adrenal hypertrophy was found in less than 20% of the animals in the air-conditioned environment, while paralytic incidence that followed intracerebral inoculation of virus exceeded 50% (Table VIII). As shown above, in such constant environment, 2/3 of the total paralytic incidence depends upon adrenal reactions induced by inoculatory trauma which takes over the role of the eliminated environmental stress.

In a stress environment, the enhancement of viral progression by inoculatory trauma seems to be reduced, as suggested by the fact that the incidence of early paralysis was significantly lower in an inconstant, than in air-

TABLE VII. Effect of Inoculatory Trauma upon Paralytic Incidence and upon Mean Incubation Period in Hamsters Receiving MEF_1 Virus Either Intracerebrally or Intraspinally.

Inoculation		No. animals	Total paralytic incidence, %	Early paralysis 1st wk, %	Late paralysis 2nd & 3rd wk, %	Mean incubation period, days
Intracer.	Intraspinal					
MEF_1 virus	None	82	39.02	28.04	10.97	6.50
None	MEF_1 virus	77	68.83	68.83	0	3.33
MEF_1 virus	S.M.B.E.*	78	66.66	48.71	17.94	6.98
S.M.B.E.	MEF_1 virus	77	62.33	55.84	6.49	4.45

* S.M.B.E. = Sterile mouse brain emulsion.

TABLE VIII. Effect of Normal Climatic Fluctuations upon Adrenal Weight, Susceptibility to Poliomyelitis Virus and Mean Incubation Period in Hamsters as Compared to Controls Maintained in a Constant Environment.

		Spontaneous adrenal hypertrophy			Susceptibility to MEF_1, intracer.			
Period of inoculation	Environmental conditions	No. animals	Average adrenal wt, mg/100 g	Proportion adrenals over 14 mg /100 g, %	No. animals	Paralytic incidence, %	Mortality, %	Mean incubation period, days
4/ 1/53-5/15	Variable*	60	17.09	86.66	139	66.90	38.84	9.39
4/ 8/54-5/14	Constant†	58	12.16	20.68	73	61.64	36.98	5.76
9/ 1/53-10/30	Variable	179	17.17	78.20	218	65.13	36.69	8.80
9/13/54-10/19	Constant	156	11.72	20.50	158	49.36	20.25	5.97
11/13/53-11/18	Variable	32	15.70	53.12	86	61.62	27.90	10.70
11/ 5/54-11/19	Constant	95	11.87	9.46	128	59.37	23.43	7.98

* A normally heated room. † Air conditioned at 23°C.

conditioned environment, despite a total paralytic incidence significantly higher in the former than in the latter (Table V). The paradoxical reduction of early paralytic cases in the fluctuating environment might have resulted from a reduction in the number of adrenal responses to inoculatory trauma, due either to temporary exhaustion of reactivity or to adaptation caused by the repeated environmental stress preceding the inoculation.

Effect of diets. A diet enriched with 1% casein hydrolysate, in addition to the normal Rockland mouse diet, increased the incidence of adrenal hypertrophy from 10% in the controls to 50% suggesting either a direct effect of the diet, or an enhancement of the adrenal reactivity to minor environmental stresses (20). The same diet enhanced significantly the paralytic rate following intracerebral inoculation of virus. As in the case with environmental stress, the casein enriched diet increased the incidence of belated paralysis to 27% from 16.76% in controls and also slightly increased the mean incubation period.

Discussion. The above findings emphasize the importance of 4 factors in the pathogenesis of experimental poliomyelitis: (a) inoculatory or environmental stress, (b) endocrine reactivity to stress, (c) persistence of inoculated virus, and (d) correct experimental timing. The effect of stress depends upon endocrine reactivity. Increased reactivity due to seasonal or dietary conditions enhances, while adaptation or temporary exhaustion of endocrine reactivity diminishes the effect of stress upon susceptibility to poliomyelitis. On the other hand, due to compensatory phases in the evolution of an endocrine disturbance, susceptibility varies with the timing of inoculation, in relation to the endocrine status present at time of viral inoculation (2). Some effects of cortisone in poliomyelitis infected hamsters give suggestive information regarding the mechanism by which the adreno-cortical hyperfunction induced by stress, may influence viral progression. Thus extensive spread and multiplication of virus in certain tissues is associated with the suppression of the non-specific inflammatory barrier produced by cortisone (1,4-9,11). Cortisone also favors accelerated and protracted viral multiplication in the brown fat cells in association with alterations in lipid metabolism(3,10,11). Experiments in progress demonstrate that stress enhances significantly the multiplication and persistence of MEF_1 virus in the brown fat of hamsters and also has a belated cortisone-like effect upon lipid metabolism. This effect upon persistence of virus may partly account for the high proportion of belated paralytic cases observed in a stress environment.

Cortisone induces viremia in hamsters by enhancing extraneural multiplication of virus, by suppressing inflammatory barriers, and by interfering with early antibody formation (1,8,9). According to Bodian, the viremia level is a main determinant of spinal cord invasion(12,13), and thus may account for the high paralytic rate observed in cortisone-treated animals.

Stress is also able to provoke viral invasion of the spinal cord. Thus, it has been shown that the paralytic incidence is significantly increased by a preliminary sterile intraspinal injection in intracerebrally inoculated, and by a preliminary sterile intracerebral injection in peripherally inoculated hamsters. A pathogen-free intramuscular injection increases paralytic rate and favors localization of the paralysis to the injected extremity of monkeys(12,13), and humans(16,17). This provoking effect of stress seems to be mediated by the resultant circulatory alterations which increase the blood supply to the corresponding part of the spinal cord(14,15) so that circulating virus can more readily penetrate into that region and set up an initial infection therein(13).

From studies thus far carried out, it may be concluded that properties like pathogenicity, invasiveness, etc., that are usually attributed to virus may actually depend on temporary alterations induced in the physiology of the host by factors as common as nutrition and environmental adaptation.

Summary. 1. The incidence of paralytic poliomyelitis in experimentally infected hamsters has been found to depend upon a variety of factors such as inoculatory trauma, environmental stress, and diet. A linear correlation was found between incidence of adrenal hypertrophy and incidence of paralytic disease induced by the above factors in 2 series of hamsters inoculated simultaneously, one with pathogen-free material, the other with MEF_1 virus. 2. Seasonal fluctuations in adrenal reactivity to the inoculatory trauma were closely associated with parallel fluctuations in susceptibility to poliomyelitis. Either, adaptation to chronic environmental stress, or rapid disappearance of the virus from certain inoculated tissues, interfered with the above correlation. 3. The mechanism by which endocrine glands and hormones may influence the progression of virus is discussed.

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1. Shwartzman, G., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 573.

2. Teodoru, C. V., and Shwartzman G., *J. Exp. Med.,* 1954, v100, 563.

3. Shwartzman, G., Aronson, S. M., Teodoru, C. V., Adler, M., and Jahiel, R., *Ann. N. Y. Acad. Sci.,* 1955, v61, 869.

4. Aronson, S. M., and Shwartzman, G., *Am. J. Path.,* 1953, v29, 381.

5. Shwartzman, G., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 835.

6. Howe, H. A., and Bodian, D., *Commonwealth Fund N. Y.,* 1942, v99.

7. Rivers, T. M., *Stanford University Press,* 1939, 42.

8. Shwartzman, G., and Aronson, S. M., *Ann. N. Y. Acad. Sci.,* 1953, v56, 793.

9. Shwartzman, G., *J. Mt. Sinai Hosp.,* 1954, v21.

10. Aronson, S. M., Teodoru, C. V., Adler, M., and Shwartzman, G., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 767.

11. Aronson, S. M., Shwartzman, G., and Teodoru, C. V., *J. Neuropath., & Exp. Neurol.,* 1955, v14, 109.

12. Bodian, D., *Am. J. Hyg.,* 1954, v60, 358.

13. ———, *Ann. N. Y. Acad. Sci.,* 1955, v61, 877.

14. Trueta, J., and Hodes, R., *Lancet,* 1954, v265, 908.

15. Levine, R., *Bull. Acad. Sci. Med.,* 1952, v8, 13.

16. Farmer, A. D., *M. J. Australia,* 1955, v1, 141.

17. Trueta, J., *Ann. N. Y. Acad. Sci.,* 1955, v61, 883.

18. Arkin, H., and Colton, R. R., *Statistical Methods,* Barnes & Noble, Inc., New York, 1953, 74.

19. Fisher, R. A., *Statistical Methods for Research Workers,* Hafner Publ. Co., New York, 1954, 206.

20. Selye, H., *J. Clin. Endocrinol.,* 1946, v6, 117.

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Host Resistance to Bacteria in Hemorrhagic Shock IV. Effect of Hypothermia on Clearance of Intravenously Injected Bacteria.* (22207)

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Dogs recover if transfused after exposure to severe hemorrhagic shock of 2 hours duration(1). But they die if given an intravenous dose of bacteria which a normal dog readily destroys(2). This is true whether the bacteria are given during the two hour period of shock or anytime within the next 24 hours. This is evidence that hemorrhagic shock impairs the bactericidal mechanisms. In this report we present evidence that precooling the animal to be subjected to hemorrhagic shock protects the bactericidal mechanisms.

Method. Healthy mongrel dogs, weighing 15-22 kg were premedicated with morphine (2 mg/kg), and then immersed in iced water under light ether anesthesia until the rectal temperature reached 28°C. After a short interval for desaturation of the ether, the dogs were removed from the iced water and put into hemorrhagic shock by the elevated reservoir technic(3). No anesthesia was required thereafter to maintain the hypothermia. During the hypotensive period the rectal temperature ranged between 28° and 23°C. In the occasional instance in which it fell below 23°C warm water bags were applied. After 2 hours in shock the shed blood was returned by slow venous infusion, during which the dog was immersed in warm water until the rectal temperature reached 37°C. 5-50 billion *E. coli* or hemolytic coagulase-positive staphylococci (24 hour culture) were given intravenously either during the period of hypotension (Group IV, Table I) or after transfusion and warming to normal body temperature (Group V, Table I). In addition to these experiments, concurrent experiments were performed to test the response of normothermic dogs, transfused after exposure to 2 hours of shock, to the same bacterial suspensions. The animals were observed until death or full recovery.

Results. These are given in Table I. The data of Groups I to III are taken from the published report of the previous work in normothermic dogs referred to above(2). The additional experiments like those in Group III, performed concurrent with those in Groups IV and V, resulted in death in every instance.

In contrast to a zero survival rate for normothermic shocked dogs challenged with bacteria, the precooled shocked dogs (Groups IV and V) challenged the same way show a survival rate of 75% or better.

Normal dogs (Group I) show negative blood cultures 24 hours after injection of the bacteria, and the tissues are sterile within 48 hours. All dogs in Group III show a bacteremia at this time and until death, which occurs within 1-4 days. Of the survivors in Groups IV and V half showed positive blood cultures after 24 hours, but all showed negative blood cultures after 48 hours. All dogs which died in Groups IV and V showed positive blood cultures until death.

Discussion. From the Group IV experiments, in which bacteria were injected during hypothermia, one could attribute the result to some degree to the suppression of bacterial activity by the hypothermia. But the Group V experiments demonstrate that whether this is so or not, the antibacterial defense is sufficiently preserved by the hypothermia to enable the dog to cope with a dose of bacteria, injected after the body temperature is back to normal, which the uncooled dog cannot withstand.

In a forthcoming publication(4) further data are offered showing that the protection conferred on the anti-bacterial defense mechanisms by this degree and duration of hypothermia, though considerable, is not suf-

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TABLE I. Effect of Hypothermia upon Resistance to Bacteria Given during or after Shock of 2 Hours Duration.*

Group	Experiment	No. of dogs	Survivors No.	Survivors %
I	*E. coli* intravenously in normal dogs	10	10	100
II	2 hr shock in normothermic dogs	10	9	90
III	2 hr shock in normothermic dogs, and *E. coli* intrav. during shock or up to 24 hr after transfusion	30	0	0
IV	Hypothermia (28°C) induced prior to 2 hr shock, *E. coli* intrav. during hypotensive period—warmed to normal temp. after transfusion	8	6	75
V	Same as Group IV except *E. coli* given after body temp. was back to normal†	14	11	79

* Hemorrhagic shock—bleeding to blood pressure 30 mm Hg for 2 hr followed by transfusion of all shed blood.

† This group includes 4 experiments in which a coagulase-positive hemolytic *Staphylococcus aureus* was used. Recovery rate is about the same for this organism as for *E. coli* in normothermic and hypothermic dogs.

ficient to fully sustain their original potency.

Conclusion. The anti-bacterial defense mechanisms of the dog, which are severely injured by exposure to hemorrhagic shock of two hours duration, are protected to a considerable degree by precooling to 28°C.

1. Schweinburg, F. B., Frank, H. A., and Fine, J., *Am. J. Physiol.*, 1954, v179, 532.

2. Jacob, S., *et al., ibid.*, 1954, v179, 523.

3. Fine, J., *et al., Bull. N. Y. Acad. Sci.*, 1952, v55, 429.

4. Friedman, E. W., Davidoff, D., and Fine, J., *Am. J. Physiol.*, in press.

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Fluorine in Urinary Tract Calculi.*† (22208)

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The pathogenesis of urolithiasis is one of the unsolved problems in modern medicine. A recent survey answered by 340 American urologists showed an average recurrence rate of 13.7%(1). It is apparent that urinary tract calculi form an important disease entity and that any new facts which may help clarify the problem of calculogenesis are worthy of attention.

The purpose of this note is to report that fluorine has been found in urinary calculi with frequency. Searching the literature failed to reveal reports of urinary calculi analyzed for fluorine. It has been reported that renal damage, albuminuria and hematuria have been found in humans and animals suffering from fluorine poisoning(7-13).

Methods. Parts of the stones from 10 cases of urolithiasis were analyzed for calcium, ammonium, magnesium, oxalates, phosphates, urates and carbonates in our laboratories. Other parts were analyzed for fluorine by the laboratories of Dr. H. Amphlett Williams of London, England, and Dr. Chester A. Snell of Foster D. Snell, Inc., N. Y. City. Determination of fluorine in such materials is a difficult, delicate and tedious laboratory procedure accomplished by different methods by each individual. The method used by Dr. Williams is that described by him in 1946(2). Dr. Chester Snell states(3) that his methods in determining fluorine content of calculi were those mentioned in the "Official Methods of Analysis

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TABLE I. Clinical and Laboratory Data.

Age	Sex	History	Location and No. of calculi	Analysis	Ca content (%)	Fluorine (parts/million) Williams	Snell
66	♂	Prostatic hypertrophy 1 yr	Recurrent vesicle (2)	Uric acid, calcium oxalate	Not done	340	
46	♀	1 yr	Ureteral (3); vesicle (2)	Calcium oxalate and phosphate	" "	790	816
80	♂	5 yr of bilateral nephrolithiasis, 1 yr vesicle calculi	Vesicle (35)	Calcium carbonate and magnesium ammonium phosphate	26.9	210	315
72	♂	2 yr prostatic hypertrophy	" (100+)	Uric acid, calcium and ammonium carbonate and phosphate (trace of magnesium)	13.6	570	850
57	♂	1 mo	Ureteral (1)	Calcium oxalate	24.9	240	520
4	♂	Congenital bilateral ureterovesicle anomalies	Bilateral ureteral and vesicle (numerous)	Magnesium ammonium phosphate	0	30	35
72	♂	Several years prostatic hypertrophy	Vesicle (numerous)	Uric acid and calcium oxalate	5.9	19	0*
62	♂	1 yr prostatic hypertrophy	Small prostatic (numerous)	Not done	26.9	730	1290
68	♂	2 yr increasing prostatic hypertrophy	Large vesicle (2)	Inner layer uric acid	0	5	0*
				Outer " "	0	4	0
37	♂	17 yr recurrent renal calculi	Ureteral (1)	Not done (previous calculi reported as calcium oxalate)	26.3	1560	1790

* Less than 1 part/million.

TABLE II. Fluorine Data from the Literature (for Comparison).

Material	Fluoride content, parts/million	Source
A. Human		
1. Normal teeth and bone	10–30	4†
2. " tissues	.8	4
3. a) 24 hr urine collection	.3–.5*	4
b) Normal urine	.4	5
4. Kidney (normal)	.78	5
5. " (in fatal fluorine poisoning)	11.6	5
6. Liver (normal)	.6	5
7. " (in fatal fluorine poisoning)	12.2	5
B. Exp. tissues from poisonings of dogs		
1. Acute fluorine poisoning	14–16	4
2. Chronic " "	2–5	4
C. Water		
1. N.Y.C. water supply	.1	14
2. Artificially fluoridated water	1.0	
3. Sea water	3.0	3
4. Naturally fluoridated water (very high content)	10 (Aztec, Ariz.)	6

* mg. † Ref.

of the Association of Official Agricultural Chemists," 7th edition. It is because of the difficulty of obtaining close correlation in results of the fluorine determinations that portions of all calculi, if available, were sent to both Dr. Williams and to Dr. Snell. Numerical differences reflect the difficulty in obtaining an accurate quantitative result in this analysis. It is also possible that fluorine content may differ at various parts of a calculus. This point will need further elucidation.

Table I gives a summary of clinical and laboratory data of 10 cases of urinary tract calculi studied.

Table II presents data from the literature, which show that values found in calculi greatly exceed those hitherto reported in biological materials including those obtained in experimental poisoning. The highest fluorine content in tissues reported in any of the papers is in the neighborhood of 30 parts per million; while in the calculi analyzed, fluorine contents up to 1500 to 1700 parts per million were found.

From the data on specimens in which calcium content could be determined, it will be noted that when calcium content is high, fluorine values are also high. In the two calculi

in which no calcium was found, fluorine content is low.

One factor that all these patients had in common was urography. Almost all patients with calculi have had either intravenous or retrograde urography usually performed with one type or another of radio-opaque medium. In order to eliminate the iodine-containing urographic dyes as the source of the fluorine found in the calculi, samples of 3 of these materials were analyzed and found to contain no fluorine.

Summary. Fluorine has been found in high concentration in 8 out of the 10 urinary tract calculi analyzed.

1. Burkland, C. E., and Rosenberg, M., *J. Urol.*, 1955, v73, 198.

2. Williams, H. A., *Analyst*, 1946, v71, 175.

3. Snell, Chester A., personal communications.

4. Gonzales, T. A., Vance, M., Helpern, M., Umberger, C. J., *Legal Medicine, Pathology and Toxicology*, 2nd Edition, 1954, Appleton Century Crofts Inc., N. Y.

5. Gettler, A. O., and Ellerbrook, L., *Am. J. M. Sc.*, 1939, v197, 625.

6. Natural Fluoride Content in Community Waters, 1954, Dept. of Health, Education and Welfare, Washington, D.C.

7. Hewelke, O., *Deutsche Med. Wehnschr.*, 1890, v16, 477.

8. Marconi, S., *Orto e Traumatol.* (Roma), 1930, No. 6.

9. Smyth, H. F., and Smyth, H. F., *J. Ind. and Eng. Chem.*, 1932, v24, 229.

10. Hauck, H. M., Steenbach, H., and Parsons, H. T., *Am. J. Phys.*, 1933, v103, 489.

11. Rabinowitch, S. M., *Canad. M. A. J.*, 1945, v52, 345.

12. Peters, J. H., *Am. J. M. Sc.*, 1948, v216, 278.

13. Hardy, H. L., *Arch. Ind. Hyg.*, 1955, v11, 273.

14. Personal communication.

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Lactic Acid Response to Epinephrine in Experimental Liver Disease.* (22209)

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Normal and partially hepatectomized rats were studied to determine whether differences in the utilization of lactic acid following the injection of epinephrine could be correlated with liver mass. Significant differences were observed and additional studies were carried out on the effect of carbon tetrachloride poisoning and bile duct ligation on the ability of rat liver to handle similar lactate loads.

Method. Sprague-Dawley albino male rats weighing 180-210 g were used. All animals were kept in a constant temperature room on a uniform diet. Animals received a subcutaneous injection of 0.02 mg/100 g body wt., of a freshly diluted solution of epinephrine with added glutathione (Adrin, Sharp & Dohme). Tail vein samples were used for lactic acid determinations. Partial hepatectomy was performed by a technic in which 70% of the liver mass was removed. Carbon tetrachloride poisoning was accomplished by the intraperitoneal injection of 0.10 cc of CCl_4/100 g body wt., a modification of a recently described technic(1).

TABLE I. Effect of Epinephrine on Blood Lactic Acid in Normal and Partially Hepatectomized Rats.

Hr after epinephrine	Normal rats	Part.-hep. rats
Resting, before epinephrine	17 ± .25*(57)	16.5± 1.3 (17)†
1	41 ± 1.7 (36)	46 ± 3.3 (16)
2	21 ± .3 (28)	37 ± 2.3 (17)
3	17 ± 2.3 (25)	36 ± 1.4 (11)
4	—	27 ± 2.3 (11)
5	—	20 ± 1.5 (13)

* S. E. † No. rats.

Results. Resting levels of blood lactic acid were the same in the normal and partially hepatectomized animals (Table I). Both

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TABLE II. Blood Lactic Acid Levels Three Hours after Epinephrine in Rats.

	No. rats	Blood lactic acid (mg %)
Normal	25	17 ± 2.3*
Part.-hep.	11	36 ± 1.4
CCl_4 2nd day	34	34 ± 3.5
" 5th "	14	20 ± 1.5
Bile-duct ligation 2nd day	8	14.5± 1
Idem 5th "	9	30 ± 2.5

* S. E.

groups exhibited a similar prompt rise in the lactic acid levels one hour after the epinephrine injection. Two hours after epinephrine, normal animals showed a rapid clearance of lactic acid and by 3 hours had returned to resting levels. Partially hepatectomized rats 1-4 days postoperatively did not return to previous resting levels of lactic acid until 5 hours after epinephrine. This difference was not present 9 days after operation. These results suggest that lactic acid determinations 3 hours after epinephrine could serve as a means of detecting liver damage. This was tested in rats by comparing CCl_4 poisoning and bile duct ligation.

The clearance of lactic acid was impaired in rats which had been injected with CCl_4 40 hours previously (Table II). These results were similar to those seen with removal of 70% of the liver mass. Determinations done 120 hours after CCl_4 poisoning, however, revealed a normal ability to handle endogenous lactic acid loads. These findings are consistent with the demonstrated regenerative capacity of the liver after CCl_4 intoxication(1).

Two days of biliary obstruction did not affect the ability to clear lactic acid in spite of obvious jaundice (Table II). Determinations done 5 days after duct ligation, however, showed impairment of this aspect of liver function probably related to the secondary cellular damage which follows prolonged bile stasis.

Discussion. When the role of the liver in lactic acid metabolism was established, investigators studied the resting blood lactic acid level in diseased liver states. Their results are equivocal(2,3). It was suggested that exogenous lactate loading might reveal impaired hepatic function, and much work has been done with this technic. These studies have proved that hepatocellular disease is detectable by this means(4,5). Few studies on endogenous lactic acid loading following epinephrine in liver disease have been reported. An early paper by Loeb *et al.* failed to show significant differences in the maximum elevation of lactic acid in patients with and without liver disease(6). Our results in rats show that while there is no difference in the maximum lactic acid elevation, there is a delayed return to normal levels following experimentally produced liver disease. These results are strikingly similar to those recorded by Nitzesco and Gontzea(7).

Theoretically the observed alteration in lactate accumulation might represent failure to destroy epinephrine by the damaged or absent hepatic tissue, since it is known that epinephrine is rapidly inactivated by the liver. In these experiments, however, the subcutaneous route of administration would minimize the opportunity for this epinephrine to pass through the liver. Furthermore, lactate disappearance curves of the same basic shape were obtained when epinephrine was injected by the intraperitoneal route despite a presumably high rate of entry into the hepatic circulation.

Summary. 1. Blood lactic acid levels were determined in rats subjected to liver damage by partial hepatectomy, carbon tetrachloride poisoning and bile duct ligation. 2. Hepatocellular loss or damage impaired the clearance rate of endogenous lactic acid after epinephrine. Biliary obstruction, *per se,* had no such effect.

1. Hoffman, J., Himes, M. B., Lapan, S., Riszki, H., Post, J., *A. M. A. Arch. Path.,* 1955, v59, 429.

2. Noah, G., *Klin. Wchnschr.,* 1927, v6, 1465.

3. Mizuno, H., *Jap. J. Gastroenterol.,* 1931, v3, 184.

4. Beckmann, K., *Klin. Wchnschr.,* 1927, v6, 2229.

5. Soffer, L., *Arch. Int. Med.,* 1937, v6, 876.

6. Loeb, R. F., Reeves, E. B., Glasier, H. P., *J. Clin. Invest.,* 1931, v10, 19.

7. Nitzesco, I. I., Gontzeu, L., *Rev. Stiint. Med.,* 1935, v24, 704.

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Comparative Thromboplastin Activity of Acetone-Dehydrated Rabbit and Human Brain.* (22210)

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The clotting factor present in various tissue extracts is commonly designated as thromboplastin and its potency is usually measured by the one-stage prothrombin time. With the development of prothrombin consumption time(1), an alternative method for estimating thromboplastic activity became available. Plasma from a severe hemophiliac which is almost completely devoid of thromboplastinogen and, therefore, of thromboplastic activity can be employed as assay medium. Prothrombin consumption in this plasma resulting from addition of various tissue extracts can be regarded as a measure of their thromboplastic activity. To make the procedure independent of hemophilic plasma, which is not always obtainable, a substitute was sought. Serum from normal platelet-poor plasma was found fairly satisfactory. It appears to contain all essential clotting factors except fibrinogen and thromboplastinogen when prepared under carefully standardized conditions. The two tissues studied were acetone-dehydrated rabbit and human brain because they are used most widely as reagents for the one-stage prothrombin time.

Methods. Prothrombin time. The method as previously described was employed and thromboplastin was prepared by means of dehydration with acetone(2). *Prothrombin consumption time.* One ml of blood was transferred to test tube which was placed in water bath at 37°C. Fifteen minutes after formation of a solid clot, the tube was centrifuged 1 minute at 2000 rpm and replaced in water bath for 45 minutes. Prothrombin remaining in serum was determined by the one-stage test previously described(3). When testing activity of thromboplastin preparations, 0.05 ml of the extract was mixed with 1 ml of hemophilic plasma (or serum obtained from normal platelet-poor plasma) and prothrombin consumption determined as before. *Serum for assaying thromboplastic activity.* Human blood collected with silicone-coated syringes and needles was transferred to centrifuge tubes also treated with silicone. The tubes were placed in ice bath for 30 minutes and then centrifuged at 3°C in an angle centrifuge for 40 minutes at 10000 rpm. Plasma which was nearly platelet-free was carefully removed and transferred to glass container placed in a water bath at 37°C. As the fibrin formed, it was promptly removed by wrapping it about a glass stirring rod. After all fibrinogen was removed, the serum was placed again in water bath at 37°C for 10 minutes and then for 30 minutes at 3°C. During this period residual thrombin becomes neutralized and thromboplastinogen disappears almost completely. No detectable diminution of prothrombin, labile and stable factors and PTC (plasma thromboplastin component) was observed. It was stored at –20°C.

Results. Thromboplastic activity measured by prothrombin time of human and rabbit brain dehydrated with acetone. It was shown previously that physiological saline extracts of acetone-dehydrated rabbit brain varying in concentration from 1½ to 5% gave a prothrombin time of 12 seconds(4).

TABLE I. Effect of Varying Concentrations of Aqueous Extract of Acetone-Dehydrated Rabbit and Human Brain on the Prothrombin Time.

	Prothrombin time, sec.			
	Rabbit brain		Human brain	
Conc. of extract, %	Normal plasma	Dicumarol plasma	Normal plasma	Dicumarol plasma
.5	14½		14	
1	14	26	13½	32
2	12	26	12	30
4	12	28	12	30
6	12	28	13	34
8	13	36	14½	44

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† Visiting Fulbright Scholar on leave from Centre Regional de Transfusion Sanguine et de Recherches Hematologiques, Montpellier, France.

TABLE II. Variation of Thromboplastic Activity in Various Types of Thromboplastin Preparations When Tested on Normal and Stable Factor Deficient Plasmas.

Thromboplastin preparation	Prothrombin time, sec.	
	Normal plasma	Plasma deficient in stable factor
Rabbit brain	12	45
Human "	12	50
Dog "	21	67
Pig "	23	68
Russell viper venom	14	13½
Commercial preparation I	11½	25
Idem II	19	27
" III	14	32
" IV*	12	50

* Made from rabbit brain.

The results recorded in Table I show that the same prothrombin time is obtained with 2 to 4% extracts of acetone-dehydrated human brain. At higher concentrations, human brain extract has a depressing effect which is somewhat greater than that of rabbit brain. This has also been observed by Stein and Abrahams(5). Both human and rabbit brain extracts give very nearly the same prothrombin times on plasma from patients receiving Dicumarol.

To compare thromboplastic activity of acetone-dehydrated rabbit and human brain with other preparations of thromboplastin, the prothrombin time was done with various types of thromboplastin using normal plasma and plasma from patient with stable factor deficiency. The results are recorded in Table II. It is to be noted that only acetone-dehydrated human and rabbit brain extracts gave comparable results on normal and stable factor deficient plasmas. Of various commercial preparations of thromboplastin, only one, which is made from acetone-dehydrated rabbit brain, gave results which were similar to those obtained on human and rabbit brain processed according to the technic of Quick.

Thromboplastic activity of acetone-dehydrated rabbit and human brain as tested by prothrombin consumption time. When increasing amounts of extracts of rabbit brain are added to plasma from a severe hemophiliac, a proportionate increase in serum prothrombin time occurs as shown in Table III. When serum, which is obtained from normal platelet-poor plasma, is substituted for hemophilic plasma, the results are very similar showing that the latter can be used successfully in place of hemophilic plasma.

On comparing the thromboplastic activity of rabbit and human brain by adding the same quantity of extract of each to the assay serum and determining resulting consumption of prothrombin, a similar but slightly greater potency was observed as shown in Table III. Brain extracts of other species have less thromboplastic activity than human and rabbit brain when measured either by prothrombin time or prothrombin consumption time and it can be seen from the results in Table IV that as prothrombin time increases, prothrombin consumption time decreases. Russell viper venom is the exception in that it produces a fairly short prothrombin time but does not cause high consumption of prothrombin. The poor thromboplastic activity of chicken brain can be attributed to species

TABLE III. Comparison of Hemophilic Plasma and Serum from Platelet-Poor Plasma as Assay Media for Thromboplastic Activity of Rabbit Brain Extract.

Conc. of rabbit brain, %	Prothrombin consumption time, sec.		
	Hemophilic plasma	Serum from platelet-poor plasma	
.0	8	8	(8)*
.2	9	9	(10)
.5	9	12	(18)
1	16	15½	(27)
2	25	20	(42)
4	34	31	(50)
6	38	35	(63)

* Figures in parentheses are the prothrombin consumption times when human brain extract was employed.

TABLE IV. Thromboplastic Activity of Various Tissue Extracts as Measured by Prothrombin Time and Prothrombin Consumption Time.

Source	Prothrombin time, sec.	Prothrombin consumption time, sec.
Rabbit brain*	12	36
Human "	12	39
Dog "	20	24
Pig "	23	19
Chicken "	57	9
Russell viper venom	14	17½

* A 2% aqueous extract was employed.

TABLE V. Effect of Heating on Thromboplastic Activity of Rabbit and Human Brain.

	Prothrombin time, sec.	Prothrombin consumption time, sec.		
		Normal platelet-poor plasma	Hemophilic plasma	Serum from platelet-poor plasma
Rabbit brain:				
Heated to 50°C	12	74	34	39
" " 60°C for 20 min.	27	28	8	15
" " " " 60 "	32	12	8	8
Human brain:				
Heated to 50°C	12	94	49	50
" " 60°C for 20 min.	16	62	18	19
" " " " 60 "	24	13	8	8

specificity since it is highly potent when added to avian blood(6).

Action of heat on thromboplastic activity of rabbit and human brain extract. No activity is lost when either human or rabbit brain is heated at 50°C but at 60°C a marked loss of activity occurs especially in rabbit brain extract(7). Human brain is more resistant to heat, and even after 20 minutes at 60°C, it still produces prothrombin consumption when added to hemophilic blood. (Table V).

Discussion. Acetone-dehydrated brain extract is admittedly a crude thromboplastin but when properly prepared either from rabbit or human brain, it has remarkably constant activity as measured by the one-stage prothrombin time. In the present study it has been shown that rabbit and human brain preparations also have almost the same activity when determined by their effect on prothrombin consumption test. Since no method is known for preparing a purified thromboplastin which has an activity equal or superior to rabbit brain, it seems desirable to accept the latter as a temporary standard of reference. Contamination with blood can be reduced to a minimum when rabbit brain is carefully cleared of visible blood vessels. The product is, therefore, devoid of labile and stable factors which shorten prothrombin time of plasmas in which these factors are deficient. The reagent is, therefore, particularly valuable when prothrombin time is employed for estimating the over-all prothrombin activity of plasma. This is especially important in controlling Dicumarol therapy since this drug diminishes both prothrombin and stable factor. High concentrations of either rabbit or human brain exert an inhibitory effect on prothrombin time but for the concentration recommended (200 mg in 5 cc saline solution), the error is negligible.

Rabbit brain extract heated to 60°C for 20 minutes looses its holothromboplastic properties but becomes a suitable reagent for differentiating hemophilia and hemophilia-like diseases from thrombocytopenia and thrombasthenia since its addition does not correct the hemophilic group but does correct the latter conditions. Human brain cannot be substituted since it is too heat-resistant.

Summary. Rabbit and human brains, when dehydrated with acetone, yield a product which has a high, constant and practically equal thromboplastic activity as measured by the one-stage prothrombin time and by prothrombin consumption time using hemophilic plasma as the assay medium. Serum prepared from normal platelet-poor plasma can be substituted for hemophilic plasma. Human brain extract differs from rabbit brain in that it has a greater inhibitory effect at high concentration and is more resistant to heat.

1. Quick, A. J., *Am. J. Med. Sci.,* 1947, v214, 272.
2. ———, *Science,* 1940, v92, 113.
3. ———, *The Physiology and Pathology of Hemostasis,* Lea & Febiger, Philadelphia, Pa., 1951.
4. Quick, A. J., and Hussey, C. V., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 732.
5. Stein, H. B., and Abrahams, O. L., *S. Afr. J. Med. Sci.,* 1954, v19, 79.
6. Quick, A. J., *Am. J. Physiol.,* 1937, v260, 118.
7. Quick, A. J., Hussey, C. V., and Stapp, W. F., *J. Lab. and Clin. Med.,* 1952, v39, 142.

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Protective Effect of Ganglionic Blocking Agents on Traumatic Shock in the Rat.* (22211)

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The effectiveness of adrenergic blocking agents in reducing the mortality in rats subjected to traumatic shock has been described (1). Local anesthetics(2) and atropine (3,4), an anticholinergic agent, also have been reported to produce significant protection from mortality resulting from total body trauma in the rat. Since ganglionic blocking activity has been described as a property of both local anesthetics and anticholinergic agents(5,6,7), an appraisal of ganglionic blocking agents for the prevention of death due to trauma appeared justified. That ganglionic blocking agents of sufficient potency and duration of action significantly reduce the mortality associated with traumatic shock in the rat has been established in the present study.

Method. The method of inducing traumatic shock was that of tumbling in rotating drums, as described by Noble and Collip(8). The method was modified in that only one shelf was used per drum and the animals' feet were individually taped. White male Wistar strain rats weighing 300 to 500 g were used. The speed of the drums was set at 40 revolutions per minute and the total revolutions recorded on a mechanical counter activated by an electronic relay. Preliminary studies indicated that mortality was directly related to the total number of turns of the drums with a lethal trauma of 50% at 800 turns. Nine hundred turns was chosen as a standard trauma level in these experiments to assure a mortality of 50% or more. The average mortality of all untreated rats (192) reported here was 77.1%. Treated animals received drug injected intraperitoneally 15 or 30 minutes prior to drumming. The compounds were dissolved in 0.9% saline and the dosages administered as mg (base or cation)/kg. Following trauma the animals were observed continuously and times of death were recorded. Any animal dying during trauma or 15 minutes thereafter was considered an immediate death and a gross autopsy was performed. Any animal evidencing rupture of a major blood vessel or a viscus was discarded. All other animals dying in the ensuing 24-hour period were considered as delayed deaths. Animals alive at the end of 24 hours were considered as surviving indefinitely. One control rat was tested with each drug-treated rat. A total of 16 animals, constituting one experiment at one dose level, were subjected to the standard trauma on the same day. Although daily mortality in untreated groups of rats varied from 50 to 100%, the susceptibility to trauma was assumed to be comparable in each group of animals subjected to drumming in one experiment. The differences in mortality between treated and control animals in individual experiments were analyzed for significance by exact probabilities for a 2 x 2 contingency table obtained from 2 times the tabulated (½ P)'s from Mainland's table (9). The over-all analysis for significance of all dose levels of each compound causing a reduction in mortality and not showing evidence of drug toxicity was analyzed by the chi-square method corrected for continuity.

Results. Table I presents the results of treatment of rats prior to trauma with 4, 5, 6, 7-tetrachloro-2-(2-dimethylaminoethyl) -indolene bis-methochloride (chlorisondamine), 3-

* Statistical evaluation of results was performed by Joseph L. Ciminera, Department of Biometrics, Sharp & Dohme.

Chlorisondamine was kindly supplied by Dr. F. F. Yonkman, Ciba Pharmaceutical Products Inc., Summit, N. J. Pentolinium was obtained through the courtesy of Dr. J. Seifter, Wyeth Institute of Applied Biochemistry, Radnor, Pa. TEA was supplied through the courtesy of Dr. A. C. Bratton, Parke, Davis & Co., Detroit, Mich. Mecamylamine was made available through the kindness of Dr. K. Pfister, Chemical Division, Merck & Co. Inc., Rahway, N. J. Hexamethonium was prepared in the Department of Organic Chemistry, Sharp & Dohme.

TABLE I. Effects of Pretreatment with Ganglionic Blocking Agents on Traumatic Shock in the Rat.

Compound	Dose, mg/kg	Controls D/T	Controls %	Treated D/T	Treated %	% survival above controls	Significance (P) Individual	Significance (P) Over-all
Chlorisondamine*	1.0	6/8	75.0	3/8	37.5	37.5	N.S.	<.001
	2.5	8/8	100.	2/8	25.0	75.0	.007	
	5.0	"	100.	3/8	37.5	62.5	.0256	
	10.0	5/8	62.5	5/8	62.5	0	—	
Mecamylamine*	1.0	7/8	87.5	7/8	87.5	0	—	"
	2.5	4/8	50.0	3/8	37.5	12.5	N.S.	
	5.0	7/8	87.5	0/8	0	87.5	.0014	
	5.0	6/8	75.0	1/8	12.5	62.5	.0406	
	10.0	5/8	62.5	3/8	37.5	25.0	N.S.	
Pentolinium*	.5	5/8	62.5	1/8	12.5	50.0	N.S.	"
	1.0	6/8	75.0	"	12.5	62.5	.0406	
	2.5	8/8	100.	4/8	50.0	50.0	N.S.	
	5.0	5/8	62.5	0/8	0	62.5	.0128	
	5.0	7/8	87.5	3/8	37.5	50.0	N.S.	
	10.0	6/8	75.0	4/8	50.0	25.0	"	
Hexamethonium†	5.0	4/8	50.0	1/8	12.5	37.5	N.S.	<.01
	10.0	6/8	75.0	3/8	37.5	37.5	"	
	20.0	"	75.0	2/8	25.0	50.0	"	
	30.0	5/8	62.5	7/8	87.5	−25.0	—	
Tetraethylammonium†	5.0	6/8	75.0	7/8	87.5	−12.5	—	
	10.0	"	75.0	6/8	75.0	0	—	
	20.0	"	75.0	"	75.0	0	—	
	35.0	8/8	100.	8/8	100.	0	—	
	50.0	"	100.	"	100.	0	—	

* Injected 30 min. prior to trauma. † Injected 15 min. prior to trauma.
D/T = No. deaths/No. tested.

methylaminoisocamphane hydrochloride (mecamylamine), pentamethylene-1:5-bis-(1-methylpyrrolidinium bitartrate) (pentolinium), 1,6-bis-(tri-methylammonium)-hexane diiodide (hexamethonium), and tetraethylammonium chloride (TEA). The specificity of these agents for producing ganglionic blockade has been established(10-14).

Significant protection from mortality induced by trauma was observed with one or more dose levels in at least two experiments with chlorisondamine, mecamylamine and pentolinium. Hexamethonium and TEA failed to provide significant protection in any one experiment. In an over-all analysis of significance for all dose levels providing protection with no evidence of toxicity, significant protection also was apparent with hexamethonium. TEA failed to provide any protection, possibly because of its known evanescent effect(15,16).

The influence of pretreatment with ganglionic blocking agents on total, immediate, and delayed deaths from drum shock is presented in Table II. These represent the pooled experimental results from the individual experiments in which statistically significant protection was produced by chlorisondamine, mecamylamine and pentolinium. While by far the greatest protection from death was apparent in those animals dying in the delayed death group, 50% reduction in mortal-

TABLE II. Reduction in Immediate and Delayed Deaths by Protective Doses of Ganglionic Blocking Agents.

Procedure	No. of rats	Mortality: Total No.	Total %	Immediate deaths No.	Immediate deaths %	Delayed deaths No.	Delayed deaths %
Control	48	40	83.3	13	27.1	27	56.2
Treated	48	7	14.5	4	8.3	3	6.2
Reduction in mortality—%			68.8		18.8		50.0

TABLE III. Increased Mortality in the Immediate Death Group from Pretreatment with Toxic Doses of Ganglionic Blocking Agents.

Procedure	No. of rats	Mortality					
		Total		Immediate deaths		Delayed deaths	
		No.	%	No.	%	No.	%
Control	56	44	78.6	10	17.8	34	60.8
Treated	56	43	76.7	31	55.3	12	21.4
± % change in mortality			−1.9		+37.5		−39.4

ity, a surprisingly significant ($P = <0.05$) reduction in mortality of 18.8% in the immediate death group also was observed.

Drug toxicity usually was evident in individual experiments from an increased mortality above the control group in those animals dying immediately, or by a decreased percentage of the treated animals surviving when the dosage range was increased, or both. Table III presents the pooled results from all individual experiments where drug toxicity was evident. A significant increase in the percentage of treated animals dying in the immediate period during or following trauma was apparent, although the difference in total mortality between treated and control rats was not significant. The decreased mortality in the delayed death group was not significant in that it represented a shift in deaths, or numbers of animals, to the immediate group.

Discussion. That pretreatment with ganglionic blocking agents produced significant protection from the mortality of drum trauma provides additional support for the observation that blockade of the sympathetic nervous system during physical assault prevents the sequence of pathologic events ultimately resulting in death(1). The role of the parasympathetic division is more difficult to assess, since the well characterized cholinergic blocking agents produce some degree of autonomic ganglionic blockade(6,7,15,17). The lack of a protective effect of atropine at lower dose ranges that effectively block the peripheral parasympathetic system indicates that postganglionic discharge of this division plays a less important part in the processes initiated by trauma. That atropine reduces mortality only when given in relatively large doses of 20 to 50 mg/kg(2,4) suggests that ganglionic blockade(6,7) was responsible for the observed protection. The protection observed from local anesthetics(2) may well, likewise, be the result of ganglionic blockade(5).

A critical dose range of the effective compounds was required to produce a significant degree of protection. Doses below the critical level provided insignificant or erratic protection. The higher dose levels tested caused a decreased protective effect due to addition of drug toxicity to the stress inflicted, even though such doses, in the absence of trauma, were below the lethal level. These observations have also been made with adrenergic blocking agents(1).

The finding that a significant reduction in mortality of those animals usually dying during trauma, or shortly thereafter, was achieved by pretreatment with ganglionic blocking agents indicates that common mechanisms may be involved in both early and delayed deaths due to trauma, or that the early deaths merely represent the more susceptible individuals in the test population. In contrast to this observation, an increase in the relative incidence of immediate deaths was noted with doses of adrenergic blocking agents that provided significant protection against drum shock(1).

The differences observed pertaining to the effects of drug treatment on early mortality between these and previous data(1) possibly are explainable on differences of duration of trauma. The duration of drumming in the studies presented was 23 minutes, as compared to 15 minutes of drum rotation (with two shelves per drum) as reported by Levy *et al.*(1).

Summary. 1. Significant protection from death due to drum shock in the rat has been demonstrated by intraperitoneal pretreatment

with the ganglionic blocking agents, chlorisondamine, mecamylamine, pentolinium and hexamethonium. 2. TEA failed to provide protection from mortality due to trauma. 3. Significant protective effects were demonstrable only at certain critical dose ranges. 4. The results provide added support to the observations that blockade of the sympathetic nervous system affords protection from the lethal effects of physical trauma.

1. Levy, E. Z., North, W. C., and Wells, J. A., *J. Pharmacol. and Exp. Therap.,* 1954, v112, 151.

2. North, W. C., and Wells, J. A., *ibid.,* 1951, v103, 356.

3. Zahl, P. A., Hutner, S. H., and Cooper, F. S., *ibid.,* 1943, v77, 143.

4. Hershey, S. G., Zweifach, B. W., and Metz, D. B., *Anesthesiology,* 1954, v15, 589.

5. Harvey, A. M., *J. Physiol.,* 1939, v95, 45.

6. Dutta, N. K., *Brit. J. Pharmacol.,* 1949, v4, 197.

7. Marrazzi, A. S., *J. Pharmacol. and Exp. Therap.,* 1939, v65, 18.

8. Noble, R. L., and Collip, J. B., *Quart. J. Exp. Physiol.,* 1942, v31, 187.

9. Mainland, D., *Canadian J. Res.,* 1948, v261, 1.

10. Trapold, J. H., Plummer, A. J., and Schneider, J. A., *J. Pharmacol. and Exp. Therap.,* 1955, v113, 51.

11. Stone, C. A., Torchiana, M. L., O'Neill, G. Q., and Beyer, K. H., *ibid.,* 1956, v116, 54.

12. Wein, R., and Mason, D. F. J., *Lancet,* 1953, v1, 454.

13. Paton, W. D. M., and Zaimis, E. C., *Brit. J. Pharmacol.,* 1951, v6, 155.

14. Acheson, G. H., and Moe, G. K., *J. Pharmacol. and Exp. Therap.,* 1946, v87, 220.

15. Hambourger, W. E., Cook, D. L., Winbury, M. M., and Freese, H. B., *ibid.,* 1950, v99, 245.

16. Moe, G. K., and Freyburger, W. A., *Pharmacol. Rev.,* 1950, v2, 61.

17. McManus, E. C., Bochey, J. M., and Beyer, K. H., *J. Pharmacol. and Exp. Therap.,* 1953, v108, 364.

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Renal Interstitial Pressure in Normal and in Anuric Man: Based on Wedged Renal Vein Pressure.* (22212)

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Knowledge of the interstitial pressure of kidneys would be of special interest in patients with acute anuria, as suggested by Peters(1) and Oliver, MacDowell and Tracy (2), that anuria is due to raised interstitial pressure caused by interstitial edema. However, this suggestion has never been substantiated by measurements in man. In dogs suffering from a renal lesion induced by clamping the renal artery for 3 to 4½ hours, the pressure recorded in a needle inserted in the renal tissue was within the normal range(3).

The method of assessing interstitial, or capillary, pressure in human tissues by wedging a cardiac catheter into small venules of an organ has been applied to lungs and liver, but never to kidneys. Such a technic is presented in this report. From the finding of a normal wedged renal vein pressure (WRVP) in acute anuria following shock it is concluded that the anuria in such cases is not caused by raised interstitial pressure.

Methods and material. With patients in the supine position a cardiac catheter No. 7-9 (in a boy of 7 yrs. No. 4) was passed under fluoroscopic guidance into a renal vein, most often the right. After entering the renal vein the catheter was advanced as far as possible into the kidney veins. When the catheter was properly wedged, pressure measurements were performed with a condenser manometer (4) connected with a cathode ray oscillograph and a photographic recorder. At beginning of

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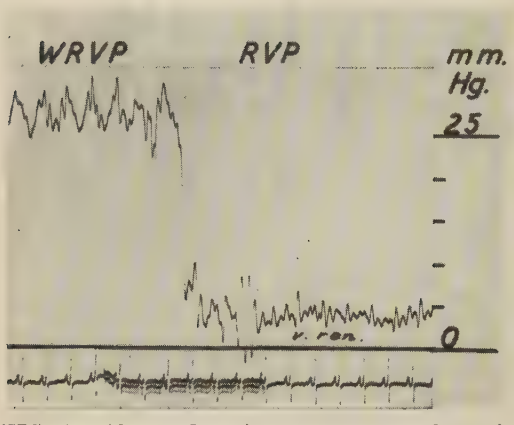

FIG. 1. Abrupt drop in pressure as catheter is withdrawn from a wedged position in renal vein to a free position.

withdrawal the catheter was stretched, followed by a sudden backward movement of the tip when released from the wedged position. Simultaneously with this characteristic slip a sudden and pronounced drop in pressure was seen together with an alteration of the configuration of the curve (Fig. 1). The highest pressure level was taken as the wedged renal vein pressure (WRVP), the lower level representing the renal venous pressure (RVP). In some cases it was impossible to obtain a pressure much higher than that in the main trunk of the renal vein. In other cases an unstable pressure was seen, presumably because the opening at the tip of the catheter was pressed against the wall of a vessel at a venous bifurcation, so that further insertion of the catheter in this position would alter the volume and pressure in the closed tube. It must therefore be emphasized that unless repeated reproduction of a pulsating curve which was stable at a high level and which showed the characteristic drop on withdrawal of the catheter, was obtainable at the oscilloscope, the examination was regarded as a failure. Besides WRVP and RVP, pressure in the right atrium and in the femoral artery were recorded. In all measurements the midaxillary line was used as zero reference. Pressure measurements were incorporated as part of renal function studies, which included renal blood flow in the anuric patients, extraction ratios of PAH and oxygen, and in some cases renal clearances(5). The 9 control subjects were patients with no evidence of renal disease or right sided heart failure. The 4 subjects with anuria or oliguria were patients who had developed acute renal failure after sustained shock following acute barbiturate poisoning (cases 3284, 3312, and 3395) or acute pancreatitis (case 3674). They were examined 5 to 7 days after the initiating shock when the peripheral circulation and blood pressure were normal, and the plasma urea concentration considerably elevated. In 2 cases follow-up studies were made.

Results. In the control group (Table I) WRVP ranged from 14-22 mm Hg, averaging 17.7. RVP ranged from 1.5-11.0, averaging 5.6 mm Hg. The right atrial pressures averaged 3.1 mm Hg, varying from –2-8. Arterial pressure averaged 125/79.

In patients examined during acute renal failure (Table II) the WRVP ranged from 15-30, averaging 20 mm Hg. RVP ranged from 5.5-15.0, averaging 10.5 mm. The right atrial pressures averaged 3.5 mm Hg, varying from 1-7. Arterial pressure averaged 128/76. In cases No. 3395 and 3674 follow-up studies showed no change in WRVP. The WRVP of 15 mm Hg given for case No. 3674 on 9th day of the disease, is from the right kidney. During the same examination a WRVP of 15.5 mm Hg was found in the left kidney.

Discussion. In the human kidney there is one central or internal system of veins—the arcuate veins—which drain the cortex as well as the medulla. Around the bases of the pyramids the arcuate veins anastomose; they unite in large branches—the interlobar veins

TABLE I. Wedged Renal Vein Pressure (WRVP) and Pressure in Renal Vein (RVP), Right Atrium and Femoral Artery in 9 Control Subjects.

Case No.	Sex	Age, yr	WRVP	RVP	Right atrium	Femoral artery
			mm Hg			
3454	♂	16	20.0	3.5	0	120/80
3509	♀	33	16.0	2.5	–2.0	"
3018	♀	19	18.5	1.5	1.0	115/70
1123	♀	30	18.5	5.0	1.0	100/65
3620	♀	32	18.0	6.5	5.5	120/70
3631	♀	40	16.0	3.0	1.0	145/90
3596	♂	27	16.5	11.0	6.0	158/84
3697	♀	46	14.0	8.0	7.0	140/90
3385	♂	7	22.0	9.0	8.0	110/80
Means			17.7	5.6	3.1	125/79

TABLE II. Pressure Measurements in 4 Patients with Acute Renal Failure Examined at Various Stages of the Disease. Symbols as in Table I.

Case No.	Sex	Age, yr	Days after shock	Days after onset of anuria	Urine flow, cc/24 hr	Plasma urea, mg %	24 hr endogenous creatinine clearance, cc/min.	WRVP	RVP	Right atrium	Femoral artery
								mm Hg			
3284	♀	41	7	5	170	444	.8	15.0	5.5	1.0	120/80
3312	♂	47	5	3	29	280	.5	30.0	15.0	5.0	162/82
			7	5	15	420	.1	18.0	13.0	—	93/70
3395	♀	27	5	4	85	380	1.0	21.0	9.0	7.0	162/85
			12	11	1740	638	4.8	22.0	10.0	10.0	182/96
			21	20	3020	46	66.0	27.0	3.5	2.0	125/82
3674	♂	51	6	5	700	405	6.0	16.5	10.0	1.0	102/63
			10	9	1680	260	33.0	15.0	10.0	—	122/80

—that run between the sides of the pyramids and the columns of Bertini to the neck of the calices, around which they form a second system of anastomoses much shorter and thicker than those at the base of the pyramids. The interlobar veins join through short thick stems into the main renal venous trunk(6,7). From necropsy studies of human kidneys we have the experience that in most cases a No. 8 cardiac catheter can be easily advanced through an interlobar vein towards its junction with the arcuate veins, the lumen of the interlobar vein being occluded by the catheter.

The pressure at the tip of a catheter occluding an interlobar vein peripherally to the set of anastomoses near the pelvis cannot be lower than the arcuate venous pressure. The pressure in an occluded interlobar vein cannot exceed the pressure in the arcuate veins, as the numerous anastomoses between these vessels are thought readily to drain away the blood which dams up behind the single catheter-blocked interlobar vein.

From these considerations it is reasonable to conclude that the WRVP as measured in this study is equal to the arcuate venous pressure, and hence very near to the pressure in the peritubular capillaries and interlobular veins. Therefore it seems justified that renal interstitial pressure can be assessed from WRVP. It is also seen that arcuate venous pressure is obtained whenever the tip of the catheter blocks the lumen of an interlobar vein between the 2 sets of anastomoses.

The normal value of 18 mm Hg for WRVP was in good agreement with values for arcuate venous pressure as measured by Swann *et al.* by renal vein catheterization in dogs; in these experiments a sudden drop in pressure of the same magnitude as observed in the present investigation (Fig. 1) was found when the catheter was withdrawn from an arcuate vein into an interlobar vein. Similarly, the present value for WRVP is nearly identical with the pressure measured in dogs by inserting a needle into the kidney(8,9,10,11).

It was impossible to demonstrate a raised WRVP in the 4 patients examined in the anuric or oliguric phase of acute renal failure. The follow-up studies in cases 3395 and 3674 deserve special interest, as they clearly demonstrate that the amount of urine excreted is independent of the WRVP.

Summary and conclusion. 1. A method is described for determination of wedged renal vein pressure (WRVP) in man. 2. In 9 normal subjects WRVP averaged 18 mm Hg. It was not possible to demonstrate a raised WRVP in 4 patients with acute anuria following shock, the average WRVP being 20 mm Hg. 3. It is argued that in agreement with experiences from lungs and liver, the interstitial pressure in kidneys equals the WRVP. The results suggest that in patients with acute anuria following shock the anuria cannot be explained by an increased interstitial renal pressure.

1. Oliver, J., MacDowell, M., and Tracy, A., *J.*

Clin. Invest., 1951, v30, 1307.

2. Peters, J. T., *Ann. Int. Med.*, 1945, v23, 221.

3. DeWardener, H. E., *Lancet*, 1955, vI, 580.

4. Hansen, A. T., *Pressure Measurements in the Human Organism*, Teknisk Forlag, Copenhagen, 1949.

5. Brun, C., Crone, C., Davidsen, H. G., Fabricius, J., Hansen, A. T., Lassen, N. A., and Munck, O., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 687.

6. Morison, D. M., *Am. J. Anat.*, 1926, v37, 53.

7. Brödel, M., *Johns Hopkins Hosp. Bull.*, 1901, v118, 10.

8. Swann, H. G., Hink, B. W., Koester, H., Moore, V., and Princ, J. M., *Science*, 1952, v115, 64.

9. Swann, H. G., Montgomery, A. V., Davis, J. C., Jr., and Mickle, E. R., *J. Exp. Med.*, 1950, v92, 625.

10. Gottschalk, C. W., *Am. J. Physiol.*, 1952, v169, 180.

11. Miles, B. E., and DeWardener, H. E., *J. Physiol.*, 1954, v123, 131.

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Action of Diethylpropanediol and Diethylbutanediol on Isolated Sciatic Nerve and Ileum.* (22213)

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2,2-diethyl 1,3-propanediol (DEP) is a central nervous system depressant remarkable for its effectiveness in protecting animals against the convulsant action of pentylenetetrazol (Metrazol). Pharmacologically, it has been characterized as an interneuronal depressant with action throughout the neuraxis but with maximal effect in the brain stem (2,3,4). 2,2-diethyl 1,4-butanediol (DEB) which differs from DEP by but one methylene group is a convulsant remarkably similar to metrazol in action(5). It too has its principal action on interneurones throughout the central nervous system with a maximal effectiveness in the brain stem. DEP protects animals from the convulsant action of DEB. The available evidence indicates that this is a non-specific example of pharmacological antagonism since other depressants and stimulants act similarly in combination with the drugs in question.

It seemed of some interest to make a quantitative comparison of the effect of these two compounds on isolated axones and synapses.

Methods. Strength-duration curves were determined for desheathed rat sciatic nerve which was maintained in Tyrode's solution at 36-37°C in a nerve chamber. The nerve was allowed to equilibrate until the curve was reproducible. Increasing concentrations of drug in Tyrode's solution were added and the strength-duration curve was determined at 15 minute intervals until concentrations causing unequivocal depression of the nerve had been added. The nerve was then washed in drug-free Tyrode's solution until a normal level of excitability had been reached. In some experiments the excitability of the nerve was enhanced by calcium ion depletion by using a calcium-free Tyrode's solution and varying concentrations of sodium citrate as suggested by Brink *et al.*(6). As a simple method of evaluating ganglionic transmission, the contraction of the isolated rabbit ileum was used following the method of Feldberg(7). To study simple neuronal pathways in the central nervous system, the segmental reflex was studied in cats in which the spinal cord had been divided at the second cervical vertebra or at both the second cervical and last thor-

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TABLE I. Comparison of Rheobase of Isolated Rat Sciatic Nerve after Soaking in Drug.

Pre-drug, mM	DEB, 100%	DEP, 100%
4	117 ± 1	120 ± 4
8	122 ± 5	124 ± 4
16	135 ± 3	140 ± 2
32	180 ± 6	182 ± 6

Mean of 8 nerves ± S.E. of mean.

acic vertebra. The last lumbar or first sacral dorsal root was divided and placed on bipolar platinum electrodes. Stimuli of 0.05-0.2 msec. duration and less than 5 volt amplitude were delivered at 10 second intervals. The response was recorded from the corresponding ventral root from platinum electrodes by a RC coupled amplifier. Each drug was tested in 6 cats, 3 with high and 3 with both high and low spinal transection. DEB was given in 1 mg/kg doses repeated at 3-5 minute intervals and effects were usually seen when about 5 mg/kg had been given. DEP was given in a dose of 100 mg/kg; occasionally a second dose of 50 mg/kg was administered.

Results. In experiments on isolated desheathed rat sciatic nerve both DEP and DEB caused a reversible increase in rheobase with little change in chronaxie. The data presented in Table I show that both drugs were effective in causing an elevation in rheobase at 4 mM. Over a wide range, 0.001 mM-2.6 mM, no concentration of either drug was found which caused an increase in excitability.

Table II summarizes the experiments in which the excitability of the nerve was enhanced by depletion of calcium ion. At doses as low as 1 mM which caused no appreciable change in the threshold of normal nerve both the anticonvulsant and the convulsant drug caused a decrease in the hyperexcitable state.

TABLE II. Comparison of Percentage Increase in Rheobase on Calcium Deficient Isolated Peripheral Nerve.

	DEB, 1 mM (%)	DEP, 1 mM (%)
Pre-calcium	100	100
Post-calcium	37 ± 12	36 ± 10
Post-drug	88 ± 9	77 ± 10

Mean of 8 nerves ± S.E. of mean.

Spontaneous firing when present stopped after drug treatment when either compound was used in a concentration of 1 mM. The more dilute solutions of DEB or DEP were without effect.

In the experiments on rabbit intestine designed to test the action of the drugs on parasympathetic ganglia, both DEP and DEB had the same depressant action. Both compounds caused a decrease in the spontaneous motility of intestinal strips. As shown in Table III these effects were comparable at various dose levels.

TABLE III. Decrease in Spontaneous Contraction of Rabbit Intestine.

Dose, mM	% reduction DEP	DEB
1	22 ± 1	18 ± 2
2	30 ± 2	22 ± 3
4	33 ± 2	26 ± 1
6	40 ± 1	40 ± 2

Mean of 6 strips ± S.E. of mean.

When 2 mM of DEB or DEP was added to the bath, 0.01 mM of nicotine or 0.4 mM/l of barium chloride failed to cause the usual increase in motility and tone. On the other hand, 0.025 mM of acetylcholine, 0.002 mM of pilocarpine or 0.005 mM of histamine still had significant stimulant effects. These findings are consistent with the view that the depression noted with DEP and DEB was indeed related to a ganglionic action.

In contrast to these results on isolated portions of the peripheral nervous system, the studies on the spinal cord confirmed the fact that DEP is a central nervous system depressant and DEB is a central nervous system stimulant. In 3 cats in which the spinal cord was divided at the level of the second cervical vertebra, DEP caused a depression of multisynaptic activity and some increase in the height of the monosynaptic spike. When the spinal cord was divided at the level of the last thoracic vertebra, DEP in each of the three cats caused a decrease in the height of both monosynaptic and polysynaptic response. Similar findings have been reported for mephenesin(8) and have been interpreted as evidence of a depression of interneural activity. The increase in the monosynaptic

spike in the high spinal animals probably represents a release from inhibition.

DEB, 5 mg/kg, had an action which resembled that of strychnine(9). The decrease in monosynaptic response in the high spinal preparation associated with an increase in multisynaptic action suggests an increase in multisynaptic inhibitory action. In the three animals with the cord divided at a low level, the stimulant action is clearly demonstrated. Thus in a preparation which represents a sample of a segment of the central nervous system little influenced by diverse inhibitory and facilitory impulses, the stimulant action of DEB and the depressant action of DEP is clearly demonstrated.

Discussion. In the experiments on isolated axones and synapses taken from the peripheral nervous system, the action of DEP and DEB was depressant. The compounds appeared to be similar qualitatively and quantitatively. It is conceivable that different tests of axonal or synaptic functions might have demonstrated some difference but we do not feel that this possibility is very great. Studies of refractory period, accommodation, period of latent addition and so on did not seem to represent a profitable line of endeavor. In support of this point of view, it can be pointed out that cocaine which is a central nervous system stimulant has been shown to have the actions on peripheral structures that are associated with the "stabilizing" group of central nervous system depressants(1). At this point it seems reasonable to suggest that for study the cellular phenomena associated with the action of drugs on the brain and spinal cord methods utilizing the central nervous system should be sought.

Summary. 1. The action of 2,2-diethyl 1, 3-propanediol (DEP), a central nervous system depressant with anticonvulsant activity and 2,2-diethyl 1,4-butanediol (DEB), a central nervous system stimulant with convulsant action have been compared on isolated synapses and axons. 2. Both compounds caused an elevation of rheobase of desheathed rat sciatic nerve at a concentration of 4 mM/l and no concentration tested caused an increase in excitability. Similarly, the treatment with each of these drugs of nerves rendered hyperexcitable with calcium ion deprivation showed only a depressant action 3. Experiments on isolated rabbit intestine showed that both compounds acted only as depressants. At 1 mM/l there was a depression of ganglionic transmission. 4. When the compounds were tested on simple reflex arcs in low spinal cats, DEP was depressant and DEB stimulatory.

1. Toman, J. E. P., *Pharmacol. Rev.,* 1952, v4, 168.

2. Berger, F. M., and Ludwig, B. J., *J. Pharmacol. Exp. Therap.,* 1950, v100, 27.

3. Slater, I. H., O'Leary, J. F., and Leary, D. E., *ibid.,* 1950, v100, 316.

4. Funderburk, W. H., and Unna, K. R., *ibid.,* 1953, v107, 344.

5. Slater, I. H., Leary, D. E., and Dresel, P. E., *ibid.,* 1954, v111, 182.

6. Brink, F., Bronk, D. W., and Larrabee, M. G, *Ann. N. Y. Acad. Sci.,* 1946, v47, 457.

7. Feldberg, W., *J. Physiol.,* 1951, v113, 483.

8. Taverner, D., *Brit. J. Pharmacol. Chemotherap.,* 1952, v7, 655.

9. Bernhardt, C. G., Taverner, D., and Widen, L., *Brit. J. Pharmacol.,* 1951, v6, 551.

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Presence of Hemolysin in Cultures of Pathogenic Leptospires. (22214)

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Hemolytic activity of a pathogenic leptospiral culture was observed in blood agar medium containing sheep erythrocytes when this culture was examined for bacterial contamination. The hemolytic potency of a "non-pathogenic" culture of water leptospires for various mammalian red blood cells had previously been reported by Sugimota(1). Except for Jungherr's casual reference to this phenomenon in reporting an outbreak of canine leptospirosis(2), no observations similar to Sugimota's has been reported for the pathogenic leptospires. Since toxic and hemolytic manifestations are frequently observed in leptospiral infections, further investigation of this chance observation was initiated by the authors—a study which demonstrated that a soluble hemolysin can be found in cultures of specific "serotype" strains of leptospires. Some characteristics of this soluble hemolysin will be described below.

Materials and methods. Preparation of hemolysin. Leptospires were cultivated in a 10% solution of inactivated normal rabbit serum (56°C for 1 hour) in isotonic phosphate buffered salt solution (pH 7.4) consisting of 0.667 g Na_2HPO_4, 0.087 g KH_2PO_4, 8.213 g NaCl, 0.190 g $MgCl_2 \cdot 6H_2O$ and 0.132 g asparagine dissolved in one liter of distilled water. Inoculated cultures were incubated 10-13 days at 30°C. Cultures were then centrifuged at approximately 4000 g, and the clear supernatant fluid removed and filtered through a Seitz E-K pad. Part of the supernatant fluid was stored in the refrigerator (5°C) and the remainder was frozen and maintained at –60°C. The sedimented leptospires were washed twice in phosphate buffer, resuspended with isotonic phosphate solution to 20% of the volume of the original culture and stored at about –60°C. For certain procedures, sedimented washed leptospires were disrupted by alternate freezing and thawing in dry-ice-alcohol and 37°C water baths, respectively. Cells were also disrupted when 20 ml aliquots of a 10-fold concentration of washed leptospires were subjected to sonic vibration at frequency of 10 kc and amplitude of 1.25 amps in a water-cooled magnetostriction oscillator. *Preparation of red blood cell (RBC) suspensions.* Sheep blood, collected by sterile venipuncture, was preserved in modified Alsever's solution(3). On the day of test, preserved cells were filtered through gauze, centrifuged at 1000 g for 10 minutes, washed 3 times with isotonic phosphate base and resuspended in a sufficient volume of base to produce approximately a 20% suspension. The concentration of cell suspensions was adjusted so that the complete lysis of a 1:40 dilution of RBC suspension had an optical density of 0.570 at 550 mμ. Erythrocyte suspensions from other animals were similarly prepared for one experiment. *Hemolysin test.* Two procedures were employed. In Procedure I, one part of a 20% sheep RBC suspension was mixed thoroughly with 9 parts of test substance in test tube and incubated in water bath adjusted to the desired temperature. After suitable intervals of time, aliquots were removed, rapidly centrifuged and diluted 1:4 with isotonic buffer. The percent hemolysis was estimated spectrophometrically at 550 mμ. In later studies this procedure was discarded and a more sensitive indicator system (Procedure II) was substituted. In this procedure, 1 ml of test fluid was mixed with an equal volume of a 1% RBC suspension in a 10 x 65 mm cuvette. After appropriate intervals of incubation, cuvettes were centrifuged and direct readings of the optical density were taken. A hemolytic unit was defined as the reciprocal of the dilution of test fluid in a 1 ml dose which results in lysis of 50% of the red cells of 1 ml of a 1% sheep

TABLE I. Comparative Hemolytic Potency of Supernatant and Cellular Fractions of *L. hemolyticus* Cultures.

Culture	Fraction*	Milieu*	% hemolysis of dilutions: Undil.	1:2	1:3	1:4	1:5	1:6	1:8	1:10	Units of hemolysin§
A	S	b + r	90	94	93	66	62	38	17	6	5.5
(16-day-old)	W (FT)†	b			93	71	48	17	8	6	.5
	W (FT)†	b + r	0	0	0	0	0	0	0	0	0
B	S	b + r	86	85	88	80	71	40	7	7	5.6
(21-day-old)	W (FT)†	b		29	0	0	0	0	0	0	<.2
	W (SV)‡	b		75	49	13	0	0	0	0	.3

* S = Supernatant; W = Washed cells; b = Base medium; r = Rabbit serum.
† 10-fold concentration of cells disrupted by successive freezing and thawing.
‡ " " " " " " sonic vibration.
§ Calculated/ml of original culture volume.

RBC suspension. The final volume of this test mixture is 2 ml. To determine 100% hemolysis and "normal" hemolysis of culture medium and diluent, suitable controls were set up with each test. *Preparation of antiserum.* A 2 kg rabbit was inoculated intravenously with successive doses of 5.0 ml, 5.0 ml, and 10.0 ml of filtered supernatant fluid at 5 to 6-day intervals. Seven days following the last inoculation, the rabbit was exsanguinated, the serum was collected, and stored at –20°C.

Results. Hemolytic studies were conducted on a strain of leptospires isolated from a human patient in Malaya. Upon antigenic analysis, the isolate was found to be a hitherto unrecognized serotype member of the hebdomadis group, and the strain was subsequently designated as *Leptospira hemolyticus* in this laboratory(4). Initial studies established the presence of a soluble hemolysin in cultures of *L. hemolyticus*. Treatment of a 2% suspension of sheep RBC with 12-day-old culture as well as with this culture's supernatant fluid, resulted in visible lysis of erythrocytes after a remarkably long incubation period of one to 2 hours at 37°C. On the other hand, a 10-fold concentration of washed leptospires prepared from the same culture and resuspended in uninoculated media demonstrated slight hemolytic activity after 6 hours of incubation. More definitive studies to determine the relative concentration of hemolysin in the culture supernatant fluid and in disrupted leptospires were conducted with two separate cultures of *L. hemolyticus*. In this procedure the more sensitive hemolysin test Procedure II was employed. The results of this study, shown in Table I, indicate that nearly all activity of *L. hemolyticus* cultures can be attributed to the soluble hemolysin present in the culture milieu. In addition, the presence of a RBC lytic inhibitor in rabbit serum is made evident by the lack of hemolytic activity of a suspension of disrupted cells in culture medium (buffered base + 10% rabbit serum). Even in the presence of RBC lytic inhibitors in cell-free liquor, the hemolytic potency of this fraction was 10- to 30-fold greater than comparable amounts of cells contained in the same volume of culture. Therefore, most of the subsequent work was conducted on the supernatant fluid.

The effect of 0°, 30°, 37°, 56°C temperatures on the hemolytic activity of the supernatant fluid, prepared from a 13-day-old culture of *L. hemolyticus,* was determined. In this study a 2% sheep RBC suspension was employed. After test tubes containing supernatant fluid were equilibrated in water baths of the desired temperatures for 10 minutes, sheep RBC were introduced and readings were taken at chosen intervals. The results shown in Fig. 1 indicate that the rate of hemolysis is considerably affected by temperature. At 0°C no hemolytic activity was manifested. The preliminary period before the onset of hemolysis, designated as the "induction period" by Bernheimer(5), was markedly reduced when the temperature was increased to 37°C. Hemolysin was completely inactivated at 56°C within 10 minutes.

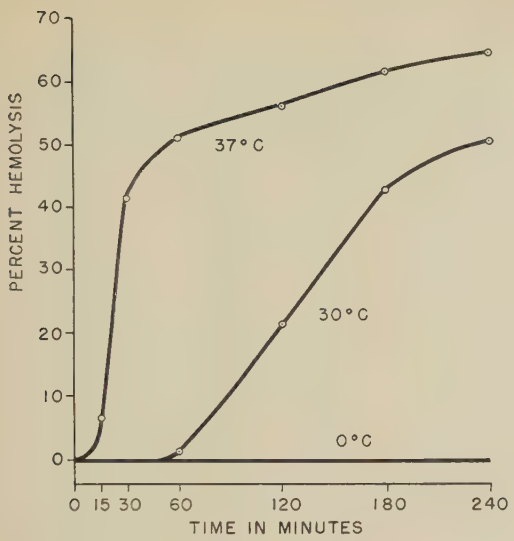

FIG. 1. Effect of temperature on course of hemolysis.

Additional studies of the culture supernatant fluid indicated that the hemolytic activity of the preparation was not reduced by continuous dialysis for 7 days against cold running tap water. No marked diminution of hemolytic activity was noted in the supernatant fraction, after this cell-free fluid was stored at 5°C (in the presence of air) or at −60°C during four months' observation. The hemolysin was not destroyed by lyophilization.

The hemolytic activity of the supernatant fraction of a culture of *L. hemolyticus* was tested against the RBC of the following species: sheep, horse, cow, goat, pig, dog, cat, chimpanzee, guinea pig, white rat, hamster, rabbit, human and chicken. Hemolysin test Procedure I was employed. Preparations were incubated at 37°C and readings were taken at the end of 2 and 4-hour intervals. Hemolytic activity was observed with sheep, cow and goat RBC only.

Forty-three different leptospiral strains representing multiple "serotypes" were screened to determine if the production of hemolysin was a generic or serotype-specific characteristic. Cell-free culture liquids were obtained from 13- to 14-day-old cultures of leptospires according to the methods previously outlined. When the spirochetes were harvested, the concentration of leptospires in each culture was determined spectrophotometrically on centrifuge-packed cells resuspended to effect a 10-fold concentrate of the culture's original volume. Supernatant preparations were treated with sheep RBC (Procedure I), incubated at 37°C, and the percent hemolysis was recorded at the 4th and 6th hour of incubation. The results of this study are shown in Table II. The minimum O. D. of any culture in which hemolysin was detected was 0.102. This figure was therefore chosen as the criterion for determining the amount of growth necessary to produce hemolysin. The results obtained with seven cultures showing lesser concentrations of leptospires were not considered to be significant. The production of hemolysin, demonstrated by the test procedures employed was restricted to specific serotype strains.

A close examination of the data in Table II revealed no apparent correlation between either the optical density or the length of the *in vitro* cultivation and the production of hemolysin by serotype strains. All strains employed were recognized "type" strains or strains identified in this laboratory through agglutinin-absorption procedures, with the following exceptions: Coke, Asbahadur, Campbell, Moulton, Perret, Williams, and Buxton. The disparity between the hemolytic reactions of various strains belonging to the same "serotype," such as, canicola, bangkinang, and hemolyticus, may reflect differences in the antigenic composition of these various strains, particularly in view of the fact that the serotype designations of some of these strains were based upon similarities in cross-agglutination lysis reaction patterns and not upon the more definitive agglutinin-absorption studies.

The results observed in various experiments indicated that the differences in hemolytic potential of *L. hemolyticus* cultures could be attributed to the age of the culture. Attempts were, therefore, made to determine the incubation period necessary to produce optimum hemolysin. A freshly seeded culture was distributed in 10 ml amounts in a

TABLE II. Hemolysis of Sheep RBC by Cell-Free Culture Liquids of Various Serotype Strains of Leptospires.

Serogroup	Serotype	Strain	Year isolated	Culture optical density	% hemolysis 4-hr	% hemolysis 6-hr
icterohemorrhagiae	ictero. AB	Wijnberg	1926	.153	—	—
	new serotype A	DeGray	54	.148	—	—
javanica	javanica	Veldrat, Bat. 46	38	.115	—	—
schüffneri	schüffneri	ML-2	51	.110	—	—
	"	Bishop	54	.105	—	—
	"	Cake*	"	.132	—	—
	"	Asbahadur*	"	.115	—	—
canicola	canicola	Ruebush	48	.168	—	—
	"	Campbell*	54	.148	16	46
	"	Moulton*	52	.125	74	85
	new serotype	Jones	54	.152	—	—
benjamin	benjamin	Benjamin	37	.250	—	47
ballum	ballum	Pasteur	–	.129	—	—
pyrogenes	pyrogenes	Salinem	24	.183	—	—
	new serotype	Biggs	54	.136	—	—
cynopteri	cynopteri	3522	38	.102	7	78
sentot	sentot	Sentot	37	.225	—	—
autumnalis	autumnalis AB	Akiyami A	25	.165	—	—
	bangkinang	Perret*	54	.108	—	—
	"	Mason	"	.148	—	55
	new serotype	Williams	"	.180	—	—
djasiman	djasiman	Djasiman	37	.249	—	—
australis A	australis A	Ballico	"	.175	73	74
pomona	pomona	LC 73*	53	.214	48	74
	"	LC 78*	54	.145	62	76
	"	V-42*	52	.118	47	74
hebdomadis	medanensis	HC	29	.192	—	—
	Wolffi A	ML-34	51	.149	—	—
	hemolyticus	Marsh	54	.270	65	78
	"	Buxton*	"	.164	—	—
bataviae	bataviae	Van Tienen	32	.127	—	85
	djatzi	HS-26	51	.169	75	85
	paidjan	Quigley	54	.135	—	45
semaranga	semaranga	RS 173	37	.248	—	—
andaman	andaman	CH 11	31	.309	—	—
biflexa		CDC	–	.315	—	—

* Serotype designation was based on cross agglutination-lysis reactions.

series of 17 x 150 mm screw cap, chemically cleaned tubes. These tubes were incubated at 30°C. At specified intervals of time, the contents of 5 culture tubes were pooled, the supernatant fraction was then harvested, and the optical density of a 10-fold concentration of cells was determined as previously described. The supernatant fluid of harvested cultures was stored at –60°C. Tests, employing hemolysin Procedure II at 37°C, were conducted on the samples. When necessary, serial dilutions of supernatant samples were tested. The hemolytic potency of each sample was expressed in units of hemolysin. The results shown in Table III indicate that the hemolysin content of actively growing cultures increases with the increased concentration of leptospires and reaches maximum values at 1-3 days following optimal growth. Continued incubation of cultures reduces the hemolytic potency to approximately one-half the maximal value by the 50th day. This decrease in hemolytic activity may in large measure be due to thermal inactivation.

Studies on the effect of concentration of hemolysin on the kinetics of sheep RBC lysis were conducted with various dilutions of a supernatant preparation that was concentrated 2-fold by pervaporation and dialysis. Hemolysin Procedure I was employed and

TABLE III. Hemolytic Activity as a Function of Culture Growth.

Age of culture (days)	Culture optical density	Units of hemolysin/cc
0	.029	<1
1	.053	<1
2	.072	<1
3	.121	<1
4	.183	1– 4
5	.203	1– 4
6	.200	8–10
7	.209	10
8	.210	10
9	.214	12–16
10	.197	16–20
12	.192	16–20
14	.157	10
16	.161	10
18	.150	10
20	.133	8–10
24	.117	8–10
48	.109	8
50	.090	8

tests were conducted at 30°C. The plotting of percent hemolysis of various dilutions of the supernatant fraction as a function of time (Fig. 2) resulted in a series of curves which did not conform to the pattern generally observed with known hemolytic systems(5).

Attempts were made to determine the presence of specific hemolysin inhibitors in rabbit antiserum prepared against supernatant fractions (agglutination-lysis titer, 1:1600). The hemolysin employed was a 20-fold concentration of washed cells, disrupted by alternate freezing and thawing. The results summarized in Table IV indicated that normal rabbit serum inhibited lysis of sheep blood cells to the same degree as antiserum.

Discussion. The clinical symptomatology of the leptospiroses has been attributed to the toxigenic manifestations of these spirochetes (6,7). Gsell(7) maintains that leptospires produce a toxin with hemolytic properties and that the severity of disease reflects the intensity of intoxication. The hemolytic manifestations of leptospiral infections in man and animals have been frequently reported (6-9). The toxic effects in guinea pigs inoculated with preparations obtained from *L. icterohemorrhagiae* cultures have been demonstrated by Fukishima and Hosoya(10), and Higuchi(11), respectively. These observations, coupled with the fact that most, if not all of the known bacterial toxin products, other than the classic exotoxins, are hemolytic(12) strongly suggest that the hemolytic activity of leptospires is an attribute of a leptospiral toxin.

An appraisal of the experimental data in the light of current concepts of leptospirosis affords an opportunity to consider several factors that may operate in differential pathogenesis of various serotype strains for particular hosts. The various serotype strains show marked over-all differences in severity of disease produced in a particular host. Thus, in man, for example, mild, frequently subclinical, infections are generally produced by *L. pomona, L. mitis, L. grippotyphosa* and *L. hebdomadis* while the more severe icteric and hemorrhagic manifestations are more frequently observed in *L. icterohemorrhagiae, L. australis A* and *L. bataviae* infections(7). In addition, particular strains show discriminate pathogenicity for various mammalian hosts as exemplified by *L. pomona* infection in rats (inapparent), hamsters (lethal), guinea pigs (inapparent), humans (rarely icteric), swine (nonicteric), and cattle (frequently icteric) (6,7,9,13). Close examination of the data in which the hemolytic potency of 36 serotype strains, including 14 very recent isolates, was tested against sheep RBC (Table II) discloses differences among serotypes with respect to this characteristic. In some instances, *e.g., L. icterohemorrhagiae,* Wijnberg, the absence of hemolysin in strains with

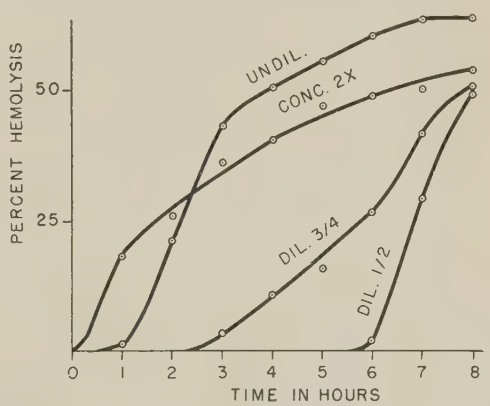

FIG. 2. Course of hemolysis of various concentrations of the supernatant fraction of a *L. hemolyticus* culture at 30°C.

TABLE IV. Comparative Titration of RBC Lytic Inhibitors in Specific Rabbit Antiserum and Normal Rabbit Serum.

Serum dilution	——Undiluted——			1:5	1:50	1:500	1:5000	—	—
" , ml	1	.6	.2	1	1	1	1	—	—
Buffer, ml	—	.4	.8	—	—	—	—	—	2
Hemolysin, ml	1	1	1	1	1	1	1	2	—
	Incubated test mixtures 30 min., 30°C								
1% sheep RBC, ml	2	2	2	2	2	2	2	2	2
	% hemolysis (1 hr 37°C)								
Antiserum	0	0	0	0	38	83	87	100	0
Normal serum	0	0	0	0	33	71	73		

a past history of "hemolytic" manifestations may perhaps be due to loss of this characteristic through continued *in vitro* subculturing over extended periods of time. In this respect, however, it is interesting to note that hemolysin was observed in 4 strains that were maintained in cultures for 17 to 23 years. The possible loss or maintenance of hemolytic properties after continued *in vitro* passage may perhaps reflect the varying degrees of adaptability of different strains to culture mediums, just as strains vary in their ability to maintain their virulence after repeated transfer *in vitro* medium. In our laboratory, a strain of *L. pomona* (S-91) was no longer lethal for hamsters after one year's passage in Fletcher's medium, while *L. djatzi* isolated from a human in Puerto Rico retained its lethality for hamsters after repeated transfers in this medium for 3 years. Van Riel observed an increase in pathogenicity for guinea pigs of an isolate after 8 months' cultivation in medium(14).

In addition to differences in the frequency of hemolysin-producing strains among heterologous serotypes, strain variation in the production of hemolysis is apparent within serotypes (Table II). Analogous observations of the differences in the pathogenicity of "homologous" serotype strains isolated from different sections of the world have been observed. The severity of *L. grippotyphosa* (bovis) infections in Israel and *L. bataviae* infection in Indonesia, are in marked contrast to the benign symptomatology produced by the respective European strains(7). On the basis of extensive studies of canine leptospirosis, Meyer *et al.*(15) concluded that—"The observations in California amply confirm the findings of Walch-Sorgdrager and Schüffner(16) that the icterogenic tendencies of *L. canicola* are a fixed property of certain strains." Stavitsky's(17) failure to substantiate the previously mentioned work of Fukishima, Hosoya and Higuchi, may be attributable to the toxigenic differences in the *L. icterohemorrhagiae* strains employed. In view of these observations, further consideration of the differences in pathogenicity of "homologous" strains may possibly be correlated in part with the hemolytic potential of these spirochetes.

The few species' RBC that are susceptible to the hemolysin of *L. hemolyticus* is particularly noteworthy. In contrast to our findings, Higuchi showed that "water leptospires" hemolysed rat, rabbit or guinea pig RBC. The data presented in Higuchi's report does not preclude the possibility that pathogenic leptospires were isolated. Whether or not this preferential activity is a function of the particular strain, reflecting perhaps the selective pathogenicity of that strain for various mammalian hosts, remains to be determined.

In large measure the activity of the hemolysin was masked by the presence of a relatively high concentration of rabbit serum (10%), necessary for the cultivation of leptospires. The inhibitory activity of normal rabbit serum on the lysis of mammalian RBC has been recognized for other hemolytic systems(18). The interfering activity of rabbit serum is demonstrated by data presented in Tables I and IV and from kinetic studies on the course of hemolysis of different concentrations of supernatant fluid (Fig. 2). The plotting of percent hemolysin as a function of time, employing different dilutions of the known bacterial hemolytic systems, elicits a typical consecutive series of sigmoid curves

with decreased slopes and increased "induction periods" as the concentration of hemolysin decreases(5). The atypical series of curves shown in Fig. 2 is attributed to the differences in the kinetics of the hemolysin and inhibitor.

Superficially, the hemolytic potency of mature cultures of *L. hemolyticus* (Table III) is low when compared to the activity of cultures of beta streptococci(5). However, the hemolytic potential of leptospires is indeed considerable when one compares the extremely low yield of approximately 25-75 mg (dry weight) of leptospires obtained per liter of culture(19) with the several grams of streptococci obtained in an equivalent volume of medium(12). In addition, the hemolytic activity of leptospires is manifested in the presence of high lytic inhibitor (10% rabbit serum) concentrations.

Although it is interesting to consider our experimental data in terms of the current knowledge of the leptospiroses, definitive interpretation of the role of leptospiral hemolysin in the virulence of leptospiral strains and host reactions to infections must be approached with caution at this time. Further study of the nature and activity of leptospiral hemolysin and its mode of formation may provide clues to a better understanding of the factors involved in the pathogenesis of leptospiral infections.

Summary. A chance observation of the hemolysis of sheep red blood cells by a culture of leptospires was investigated. Studies were conducted on a newly disclosed serotype strain, isolated in Malaya and designated as *Leptospira hemolyticus*. The presence of a soluble non-dialysable, thermolabile, oxygen-stable hemolysin in the supernatant fluid of cultures was demonstrated. The optimum temperature for hemolytic activity was 37°C. At lower temperatures the activity of hemolysin progressively decreased. The hemolytic activity against sheep RBC occurred after a prolonged induction period. This phenomenon as well as the atypical kinetic activity of varying concentrations of hemolysin preparations were attributed to the inhibitory activity of the rabbit serum present in culture supernatants. The production of hemolysin was not a generic characteristic but was restricted to specific serotype strains. Hemolytic activity of *L. hemolyticus* was manifested against sheep, cow, goat RBC, but not against RBC of eleven other species screened. The antigenicity of this hemolysin could not be demonstrated. In actively growing cultures, maximum amounts of hemolysin were produced 1 to 3 days following optimum growth. The presence of a soluble hemolysin in specific serotype strains may figure significantly in explaining the pathogenicity of leptospiral infections.

1. Sugimoto, H., *Jap. J. Exp. Med.*, 1954, v16, 143.

2. Jungherr, E., *J. Am. Vet. Med. Assn.*, 1937, v91, 661.

3. Bukantz, S. C., Rein, C. R., and Kent, J. F., *J. Lab. and Clin. Med.*, 1946, v31, 394.

4. Unpublished data from Division of Veterinary Medicine, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C.

5. Bernheimer, A. W., *J. Gen. Physiol.*, 1947, v30, 337.

6. Thiel, P. H. van, *The Leptospiroses,* Universitaire pers Leiden, Leiden, 1948.

7. Gsell, O., *Leptospirosen,* Verlag Hans Huber, Bern, 1952.

8. Walch-Sorgdrager, B., *Bull. Health Org., League of Nations,* 1939, v8, 143.

9. Reinhard, K. R., *Exp. Parasitology,* 1953, v2, 87.

10. Fukushima, B., and Hosoya, S., *Sci. Rep. Gov't. Inst. Infectious Diseases, Tokyo Imp. Univ.,* 1926, v5, 151.

11. Higuchi, S., *Fukuoka Acta Medica,* 1931, v24, 3.

12. Bernheimer, A. W., *Bact. Rev.*, 1948, v12, 195.

13. Gochenour, W. S., Jr., Yager, R. H., and Wetmore, P. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 199.

14. Riel, J. van, *Le Foyer Centro-Africain de Leptospirose, Ann. Soc. Belge de Med. Trop.*, 1946.

15. Meyer, K. F., Stewart-Anderson, B., Eddie, M. A., *J. Am. Vet. Med. Assn.*, 1939, v95, 710.

16. Walch-Sordrager, B., and Schüffner, W., *Zentbl. Bakt.*, Abt 1, Orig. 1938, v141, 97.

17. Stavitsky, A. B., *J. Inf. Dis.*, 1945, v76, 179.

18. Stollerman, G. H., Bernheimer, A. W., and MacLeod, C. M., *J. Clin. Invest.*, 1950, v29, 1936.

19. Hiatt, C. W., *Symposium on the Leptospiroses,* Medical Science Publ. No. 1, 1953, 154.

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Inactivation of Some Animal Viruses with Gamma Radiation from Cobalt-60.* (22215)

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The need for obtaining better methods of inactivating viruses for preparing non-infectious vaccines and other biologicals indicates the desirability of exploring more efficient means of virus inactivation. While some information has been accumulated concerning the effect of small doses of alpha, beta and gamma radiation on viruses(1), little data that deal with sterilizing doses are available. Huber(2) has reported that many virus preparations can be inactivated by 40000 to 1400000 roentgen equivalent physicals (rep), using high intensity electron beams from a capacitron. Nickerson(3) has indicated considerably higher sterilization doses for viruses, varying from 1 to 20 million rep.

Studies described here deal with the effect of gamma radiation on 4 animal viruses *in vitro*. The experiments were designed to determine the dosage necessary to render these viruses non-infectious when irradiated in whole brain and in suspension. Since it has been suggested(1) that a relationship may exist between size of viruses and their resistance to ionizing radiations, the viruses used were selected to cover a range of particle size.

Materials and methods. At the time this study was initiated, poliovirus was selected as representative of a small virus. St. Louis encephalitis (SLE) and Western equine encephalomyelitis (WEE) viruses were chosen to represent medium sized viruses, and vaccinia a large virus. More recent data, however, indicate that poliovirus is somewhat larger than was previously believed and approximates the size of St. Louis virus(4). *Viruses* used and their approximate diameters were as follows: California strain of WEE virus 53 mμ(5), Hubbard strain of SLE virus 20-30 mμ(6) obtained from American Type Culture Collection. Armstrong 1166 neurotropic strain of vaccinia virus 225 mμ(7), Lansing strain of poliomyelitis virus 22 to 27 mμ diameter(4) were kindly supplied by the University of Michigan School of Public Health. All *virus suspensions* were prepared from infected brains of 3- to 4-week-old Swiss mice. Ten percent brain suspensions were prepared by grinding with alundum and cold physiological saline. Suspensions were subjected to the following cycles of treatment: (a) sedimentation at 2000 rpm for 5 minutes; the supernatant fluid was used as crude virus (CV), (b) sedimentation of CV at 3000 rpm for 10 minutes, (c) sedimentation of second supernatant fluid at 4000 rpm for 15 minutes, (d) filtration of third supernatant fluid through a Seitz EK pad, (e) sedimentation of the filtrate at 45000 rpm for 1.5 hours and reconstitution of the pellet in pH 7.2, 0.05 M phosphate buffer, (f) sedimentation of the reconstituted material at 4000 rpm for 15 minutes. Alternate high and low speed centrifugations were repeated until the virus preparation had been washed 3 times. Immediately after the final wash the virus pellet was resuspended in 2 ml of phosphate buffer to make a concentrated suspension of partially purified virus (PPV). One ml aliquots of all virus suspensions were placed in glass plastic-capped vials and shell frozen in a dry-ice-alcohol bath. Vials containing the frozen virus were then stored at −50°C until they were irradiated. Samples of whole mouse brains were frozen and stored in the same manner. *Titration of viruses.* The various virus suspensions were titrated for infectivity by intracerebral injection of 3- to 4-week-old mice using .05 M phosphate buffer as diluent. The LD_{50} titers were determined by the method of Reed and Muench(8). The various virus

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TABLE I. Rates of Inactivation of Poliovirus, St. Louis Encephalitis, Western Equine Encephalomyelitis and Vaccinia Viruses in Whole Brain, Crude Suspensions and Partially Purified States.

Virus	Diameter, mμ	Virus sample	Unirradiated controls	rep* radiation in millions							
				1	1.5	2	2.5	3	3.5	4	4.5
Poliovirus	22–27	WB†	4.3‡	3.3	3.0	2.3	1.9	1.3	0	0	0
		CV	6.0	4.6	4.0	3.3	2.6	2.2	1.4	0	0
		PPV	6.0	4.2	3.3	2.5	1.7	0	0	0	0
SLE	20–30	WB	7.2	5.8	5.1	4.3	3.5	3.1	2.2	1.5	0
		CV	6.0	4.6	4.0	3.3	2.7	2.1	1.4	0	0
		PPV	6.0	4.2	3.3	2.5	1.6	0	0	0	0
WEE	53	WB	8.1	6.3	5.4	4.8	4.0	3.2	2.3	1.6	0
		CV	6.0	4.4	3.6	3.1	2.2	1.6	0	0	0
		PPV	6.0	4.0	2.8	2.0	0	0	0	0	0
Vaccinia	225	WB	5.2	3.7	2.6	1.8	0	0	0	0	0
		CV	6.0	4.1	3.0	1.9	0	0	0	0	0
		PPV	6.0	2.5	0	0	0	0	0	0	0

* Roentgen equivalent physicals. † WB—whole brain, CV—crude virus, PPV—partially purified virus. ‡ All figures represent log of LD_{50} dilution.

suspensions were diluted to desired LD_{50}, just before they were irradiated. *Method of Irradiation.* Samples were removed from the deep freeze and placed in the center well of the large cobalt-60 source housed in the Fission Products Laboratory of the University of Michigan. Exposure rate of gamma rays emitted was approximately 200000 roentgen equivalent physicals (rep) per hour(9). One rep as defined for tissue in air represents an energy absorption dose of 93 ergs/g. Ferrous-ferric dosimetry was used for calibration. The calibration solution was placed in vials similar to those used for virus preparations and readings were based on oxidation of 15.4 μ moles of ferrous ions/liter/1000 rep. Uniform doses were attained by rotating samples on a horizontal plane in the center well of the Cobalt source. Samples to be tested were irradiated from 5 to 24 hours, during which time they were kept frozen with dry ice at approximately −72°C. All virus samples to be irradiated for a given experiment were placed in the center well at the same time. After the required exposure, samples were removed and immediately tested for presence of virus by intracerebral titration in mice, using 10 mice for each of an appropriate series of 10-fold dilutions. Control samples of virus were exposed to identical conditions but were not irradiated.

Results. As a first step toward studying the effect of gamma rays on animal viruses, samples of virus preparations were exposed to cobalt-60 radiations from 1 to 4.5 million rep. Frozen CV and PPV suspensions diluted (to the same volume) in 0.05 M phosphate buffer to contain the same number of LD_{50}, and whole brain preparations were irradiated under conditions described. During irradiation, samples of virus were removed at intervals computed to yield doses of gamma rays as indicated in Table I. Each sample was tested for reduction in virus titer by intracerebral injection in mice.

It may be seen that WB preparations for all viruses tested required longer periods of irradiation than did either CV or PPV suspensions. Crude suspensions of all viruses were inactivated at a significantly faster rate than were PPV suspensions. Results obtained with SLE virus are almost identical to those obtained with poliomyelitis virus. Comparison of these data show that both SLE and poliovirus required 3.5 to 4 million rep before CV suspensions were rendered non-infectious, and that PPV samples lost their infectivity at 2.5 to 3 million rep. From data presented it is evident that crude suspensions of WEE virus required less irradiation before infectivity was lost than did comparable suspensions of SLE or poliovirus. Similarly, PPV suspensions of Western virus were reduced in infectivity at a faster rate than were the SLE and Lansing viruses. Comparison of data on PPV suspensions reveals that West-

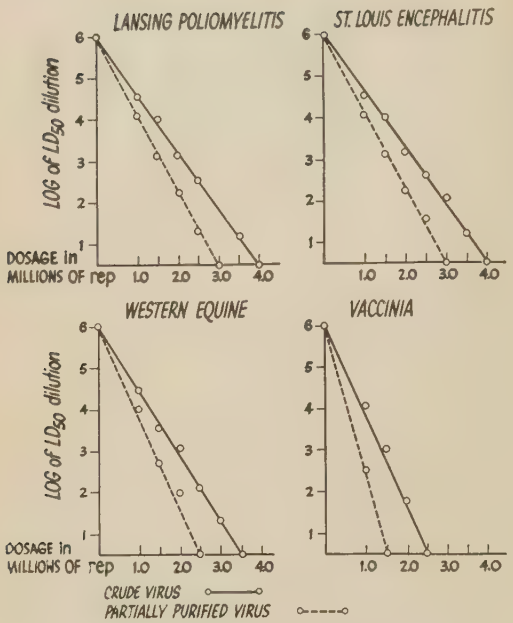

FIG. 1. Survival curves of gamma irradiated viruses in crude and partially purified suspensions.

ern PPV was no longer infectious after 2.5 million rep of irradiation. Partially purified SLE and poliovirus, on the other hand, required an additional 500000 rep before suspensions lost their pathogenicity for mice. It appears that inactivation of vaccinia virus by gamma radiation followed the same general sequence as that described for SLE, WEE, and poliovirus.

To show graphically the rates of inactivation for CV and PPV suspensions containing the same number of LD_{50}, the data in Table I were plotted on semi-logarithmic paper and presented in Fig. 1. The points on these curves are averages of virus dilution expressed as logarithms of the LD_{50}. The slopes of the curves suggest that purification of viruses removed some substance which protected the virus particle from the lethal effects of gamma rays. When the surviving virus fractions, or LD_{50}, are plotted on a logarithmic scale, the points are found to lie approximately on a straight line showing that, within error of the experiment, the surviving fraction is an exponential function of the dose. It should be pointed out that although error of assessment of virus activity may sometimes be rather large, there appear to be no constant deviations from exponential survival as distinct from random variations.

Discussion. The relationship between virus size and dose of gamma radiation required for inactivation is seen in Table I. The data show that CV suspensions of poliomyelitis and SLE virus required the same dosage of radiation before they were inactivated, while vaccinia virus was rendered non-infectious by a significantly smaller dose. Inspection of the data obtained with PPV suspensions suggest that smaller virus particles require larger doses of radiation for inactivation than do larger virus particles. It appears that the differential in size between poliovirus and SLE is not sufficient to make a difference in the dose of cobalt radiation needed for inactivation.

Of the PPV suspensions tested it may be seen that SLE and poliovirus with diameters of 20-30 mμ required 3 million rep for complete inactivation while the intermediate WEE virus required a slightly smaller dose. Vaccinia, the largest virus used, was inactivated with half the radiation required for the smallest viruses.

Preliminary experiments indicate that 2 to 3 times the dose of cobalt-60 required for inactivation does not impair the antigenicity of any of the viruses tested, and that gamma irradiated antigens elicit an immunologic response comparable to that induced by chemically inactivated viruses. These experiments will be presented in detail in a subsequent report.

Summary. 1. Gamma radiation from cobalt-60 proves to be an effective method for inactivation of Lansing poliomyelitis, St. Louis encephalitis, Western equine encephalomyelitis and vaccinia viruses. 2. Partially purified suspensions of viruses tested were more vulnerable to lethal effects of gamma radiation than were crude suspensions. 3. In crude and partially purified suspensions the smaller viruses required larger doses of gamma radiation for inactivation than did the larger viruses. 4. The rate of inactivation for viruses tested, within error of experiment,

is an exponential function of the dosage of gamma radiation.

1. Lea, D. S., *Actions of Radiations on Living Cells,* Cambridge Press, 1955, Chap. IV, 100.

2. Huber, W., *Ann. N. Y. Acad. Sci.,* 1952, v55, 536.

3. Nickerson, J. T. R., Proctor, B., and Goldblith, S. A., *Am. J. Pub. Health,* 1953, v43, 554.

4. Schwerdt, C. E., and Schaffer, F. L., *Ann. N. Y. Acad. Sci.,* 1955, v61, 740.

5. Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *Arch. Path.,* 1943, v36, 167.

6. Elford, W. J., and Perdrau, J. R., *J. Path. Bact.,* 1935, v40, 143.

7. Green, R. H., Anderson, T. F., and Smadel, J. E., *J. Exp. Med.,* 1942, v75, 651.

8. Reed, L. J., and Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

9. Lewis, J. G., Nehemias, J. V., Harmer, D. E., and Martin, J. J., *Nucleonics,* 1954, v12, 40.

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Infection of *Mansonia perturbans* and *Psorophora ferox* Mosquitoes with Venezuelan Equine Encephalomyelitis Virus. (22216)

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Past studies have indicated Venezuelan equine encephalomyelitis (VEE) to be a mosquito-borne disease. In Trinidad, Gilyard(1) transmitted VEE from donkey to donkey by bite of *Mansonia titillans* (Walker). In Ecuador, Levi-Castillo(2) reported transmission by *Aedes taeniorhynchus* (Wied.), *M. titillans,* and *Culex quinquefasciatus* (Say). Recent work in the United States(3-5) has shown *A. triseriatus* (Say) to transmit efficiently in the laboratory.

The present report concerns studies on the VEE vector potentials of two additional species of mosquitoes, *M. perturbans* (Walker) and *Psorophora ferox* (Humboldt).

Materials and methods. The strain of VEE virus used, and methods of virus titration and of handling, feeding, incubating, and processing the mosquitoes were the same as reported previously(3,4). The *M. perturbans* were collected alive in Louisiana as adults, and were used in experiments 5 days later. The *P. ferox* were reared from larvae collected near Montgomery, Ala., and were 7-10 days old when used. Adult guinea pigs inoculated intraperitoneally with 1,000-10,000 mouse LD_{50} of virus 48 hours previously served as sources for mosquito infection. After incubation at 80°F, the mosquitoes were refed individually upon normal 3-week-old guinea pigs to determine their ability to transmit infection by bite.

Results. M. perturbans. Forty females engorged upon blood having a mouse intraperitoneal virus titer of $10^{8.4}$. A total of 16 egg rafts were laid by these specimens 6-13 days later. These rafts were ground in 2 ml of diluent each and titrated for virus. On the 13th day of incubation 16 of 29 mosquitoes remaining alive refed individually upon normal guinea pigs. These mosquitoes, as well as those which did not refeed, were then titrated to determine the amount of virus each contained. Table I summarizes the infection and transmission data.

P. ferox. Fifty females were fed upon blood containing virus titering $10^{6.3}$. Several hundred eggs laid 6-11 days later were pooled, ground in 2 ml of diluent, and inoculated into mice to test for virus.

On the 14th day of incubation the surviving mosquitoes were given opportunity to refeed individually upon normal guinea pigs. Subsequently, all mosquitoes were titrated to determine the amount of virus contained per

TABLE I. Infection of *M. perturbans* and *P. ferox* with VEE Virus.

Species	Titer of virus in blood ingested (mouse i.p. LD_{50})	% mosquitoes infected	Mouse i.p. LD_{50} contained/infected mosquito	% mosquitoes transmitting by bite	Virus recovered from mosquito eggs
M. perturbans	$10^{8.4}$	83 (24/29)	$10^{3.9}$ to $10^{6.0}$	38 (6/16)	+, 7 of 16 rafts*
P. ferox	$10^{6.3}$	84 (32/38)	$10^{1.5}$ to $10^{5.2}$	11 (2/18)	—

* No more than 300 mouse i.p. LD_{50} of virus were recovered from any of the rafts.

specimen. Infection and transmission results are included in Table I.

Discussion. The finding of virus in 7 of 16 *M. perturbans* egg rafts is of interest but as yet of unknown significance. Inability to rear this species in the larval stage prevented determining whether a second generation of infected females could be reared from these eggs. A similar study with eastern equine encephalomyelitis in *M. perturbans* has shown 2 out of 19 egg rafts laid following an infected meal to contain virus(6). It is possible, of course, that the virus may have been located only on the surface of the eggs despite rinsing with serum-saline solution. However, these findings are reported here in the hope that they may stimulate a more complete investigation.

The infection and transmission rates show *M. perturbans* and *P. ferox* to be susceptible to VEE infection and capable of transmitting it. Both of these species are abundant and widely distributed in the United States and are fierce biters upon man. Therefore, they must be considered as potentially dangerous vectors of VEE infection should this virus be introduced into the United States.

Summary. Two species of mosquitoes, *Mansonia perturbans* (Walker) and *Psorophora ferox* (Humboldt), were allowed to feed upon guinea pigs infected with the virus of Venezuelan equine encephalomyelitis. After 2 weeks incubation at 80°F, the infection rate and guinea pig transmission rate for *M. perturbans* were found to be 83% and 38%, respectively, and for *P. ferox*, 84% and 11%, respectively. Virus was isolated from 7 of 16 *M. perturbans* egg rafts, but not from the eggs of *P. ferox*. The significance of the infected eggs is still unknown.

1. Gilyard, R. T., *Bull. U. S. Army Med. Dept.*, No. 75, 1944, p96.

2. Levi-Castillo, R., *Acta Trop.*, 1952, v9, 77.

3. Kissling, R. E., Chamberlain, R. W., Nelson, D. B., and Stamm, D. D., *Am. J. Hyg.*, in press.

4. Chamberlain, R. W., Kissling, R. E., Stamm, D. D., and Nelson, D. B., *ibid.*, in press.

5. Corristan, E. C., unpublished data.

6. Chamberlain, R. W., unpublished data.

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Host Resistance to Bacteria in Hemorrhagic Shock V. Mobilization of Phagocytes.* (22217)

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We have given evidence that hemorrhagic shock reduces efficiency of the phagocytosis-promoting factor in serum(1), increases sensitivity to bacterial toxins(2), and decreases the Properdin activity of serum(3). This report deals with the effect of hemorrhagic shock on the capacity to mobilize phagocytes to the site of challenge.

Method. 50 cc sterile beef infusion broth was injected intraperitoneally into rabbits immediately after transfusion for hemorrhagic shock of 2 hours duration. The infusion was also given at the same time to normal rabbits of the same weight and litter. Six hours later, after white blood count was made, both groups were killed by exsanguination. The peritoneal cavity was opened immediately thereafter, and cellular exudate recovered.† For this purpose the abdominal incisions were fashioned to produce a trough into which gelatin-Locke's solution at 4°C was introduced and aspirated after a short interval of gentle but thorough prodding of the immersed viscera. Washing and aspiration was continued until a total of 500 ml of Locke's solution was used. The recovered fluid was then spun in refrigerated centrifuge, and the precipitate washed 3 times in Locke's solution to get rid of fibrin and fibrin clots. The number of cells recovered was counted, and the percent of different types of cells determined from a count of 200 cells in stained smears. To compare number of cells mobilized in normal and shocked animals in response to the stimulus of the injected broth, it was necessary to obtain control data on number of cells that can be obtained by the same peritoneal washing technic from peritoneal cavities of normal and shocked rabbits of the same weight and litter, into which nothing has been injected. The shocked rabbits of this group were also killed 6 hours after transfusion.

Results. (Table I) A. *Control Data.* 1. *Normal rabbits—no intraperitoneal injection.* Number of cells recovered from 6 normal rabbits averaged 3.3 million (range 2.7-3.7 million). 65% of these were large mononuclear cells, 15% were lymphocytes and 20% were polymorphonuclear leucocytes.

2. *Shocked rabbits—no intraperitoneal injection.* Number of cells recovered from 6 shocked rabbits averaged 3.9 million (range 2.5-4.9 million). 75% of these were large mononuclear cells, 5% were lymphocytes, and 20% were polymorphonuclear leucocytes. The large mononuclear cells from both normal and shocked rabbits were elongated, somewhat flattened cells showing amoeboid activity. The fact that the number of cells in both groups was about equal, and that most of them were an apparently early form of the mature macrophage, suggests that they were for the most part traumatically desquamated peritoneal lining cells.

It is noteworthy that whereas no bacteria were observed in the cells from normal rabbits, ingested bacteria were seen occasionally in cells from 3 of the 6 shocked rabbits.

B. *Cellular Response 6 Hours After Intraperitoneal Injection of Beef Infusion Broth.* 1. *Normal rabbits.* The number of cells recovered from a series of 24 animals averaged 103 million (range 32-229 million). Of these 93% (range 85-98%) were polymorphonuclear leucocytes and 7% (range 1.5-2%) were macrophages, of which about half were mature, and the other half immature, *i.e.* like those recovered from the control animals. No bacteria were seen in stained smears of these cells. The white blood count in these rabbits

* Aided by a grant from the National Heart Institute (H-2014).

† Sterile technic was employed in this and all further procedures where indicated.

TABLE I. Response of Normal Rabbits and of Rabbits Transfused after Two Hours Hemorrhagic Shock to Intraperitoneal Beef Infusion Broth Six Hours after Injection.

Intraper. stimulus	Cellular response in normal rabbits				Cellular response in shocked rabbits				Remarks
	% of				% of				
	Macrophages	Polys	Lymphocytes	Total in millions	Macrophages	Polys	Lymphocytes	Total in millions	
None	65*	20	15	3.3	75*	20	5	3.9	Bacteria in 3 of 6 shocked rabbits. None in unshocked rabbits.
Beef infusion broth	7†	93	—	103	75*	25	—	4.2	Twice as many bacteria as cells in 8 of 18 rabbits. None in unshocked rabbits.

* Chiefly immature macrophages. † 50% mature macrophages.

just before they were killed varied from 5800-6300 per cu mm.

2. *Shocked rabbits.* Twenty-four rabbits were prepared. Of these 6 died before the sixth hour following transfusion. Number of cells recovered from the 18 rabbits killed 6 hours after transfusion averaged 4.2 million (range 1.8-8.0 million if we except one from which 30 million were obtained). 75% of these (range 60-80%) were macrophages, most of which were immature, and 25% (range 40-20%) were polymorphonuclear cells. Just before these rabbits were killed their white blood cell counts were 3700-7600/ cu mm.

In 8 of the 18 rabbits Gram positive and Gram negative bacilli and diplococci were found in considerable numbers.‡ There were about twice as many bacteria as cells per oil immersion field. Rather more than half of the bacteria were extracellular and the remainder intracellular. In addition *Coccidia* were observed in the exudate of 3 of the shocked rabbits.

Discussion. The considerable cellular response of the normal rabbit's peritoneal cavity to beef broth infusion demonstrates the irritant properties of this material. Since the number of cells that are recovered from the irritated peritoneal cavity of the shocked rabbit does not exceed the number recovered from the non-irritated cavity of the shocked rabbit, it is plain that shock severely impedes the mobilization of phagocytes, as Mahoney (4) and Miles and Niven(5) have demonstrated. That the cellular response should be poor *during* oligemic shock is not surprising, for the hemodynamic obstacle to mobilization is clear enough. But one would not expect this response in quantitative terms to be virtually zero during a 6 hour interval after restoration to a presumably normal hemodynamic status, in view of the fact that the white blood counts just before killing are about equal in normal and shocked rabbit. Since the normal animal's circulation delivers

‡ When clean but non-sterile technic was employed in harvesting the peritoneal exudate from normal rabbits, the only bacteria that we could recover were an occasional *B. sutilis*.

an average of 102 million polymorphonuclear leucocytes to the area of challenge, whereas the shocked animal's circulation delivers very few if any, the failure of these leucocytes in the shocked animal to enter area of challenge during post transfusion period appears to be due to some factor related to vascular permeability to these cells, rather than to a hemodynamic deficiency. This suggests that a mechanism such as the one recently described by Miles and Wilhelm(6), *i.e.* a cell permeability-promoting factor in serum, may have been inactivated by the shock process.

Another index of functional failure of the antibacterial defense system as a result of shock is inability of peritoneal lining cells and other precursors in the shocked animal to respond to the challenge for more macrophages, for the macrophages that are recovered are not increased in number, and of those recovered, most are still of the immature variety. This response contrasts with that of the normal rabbit in which the number of macrophages is about doubled, and about half of them are of the mature variety.

No bacteria were found in or outside the phagocytes in normal rabbit's exudate. But at least three varieties of bacteria, in a 2:1 proportion to cells, were found both in and outside the cells of the shocked rabbit.§ Since the stimulus to migration of bacteria from the intestine cannot be considered to be any less in the normal than in the shocked rabbit, the presence of bacteria in the shocked animal is further and unequivocal evidence of collapse of the antibacterial mechanisms in shock.

Summary and conclusion. When the peritoneal cavity of the rabbit recovering from mild shock in response to transfusion is challenged by an irritant fluid, the cellular response as compared to that in a normal rabbit is extremely poor. The failure to mobilize polymorphonuclear leucocytes is, for the most part, not due to defective hemodynamics, but to a disturbance of vascular permeability to migrating leucocytes. The failure to supply additional macrophages at the site of the irritation appears to be due to a defect in the cellular processes involved in the conversion of their precursors. The presence of a substantial intracellular and extracellular bacterial population in the peritoneal fluid of the shocked animal, and the absence of bacteria in the peritoneal fluid of the unshocked animal, is further evidence of the loss of resistance to bacteria in the shocked animal, and of its persistence after restoration of a normal hemodynamic status.

§ Previous studies indicate that the source of these bacteria is the intestine and that route of invasion is by transmural migration(7).

1. Schweinburg, F. B., Yashar, Y., Aprahamian, H. A., Davidoff, D., and Fine, J., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 587.

2. Schweinburg, F. B., and Fine, J., *ibid.*, 1955, v88, 589.

3. Frank, E. D., Fine, J., and Pillemer, L., *ibid.*, 1955, v89, 223.

4. Mahoney, E. B., Howland, J. W., and Yackel, K., *Surgery*, 1945, v17, 805.

5. Miles, A. A., and Niven, J. S. F., *Brit. J. Exp. Path.*, 1950, v31, 73.

6. Miles, A. A., and Wilhelm, D. L., *ibid.*, 1955, v36, 71.

7. Schweinburg, F. B., Seligman, A. M., and Fine, J., *New Eng. J. Med.*, 1950, v242, 747.

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Effect of a High Phosphorus Diet on Acid-Base Balance in Guinea Pigs.* (22218)

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The injurious effect of high phosphorus diets for the guinea pig has been reported (1,2). The animals grew slowly, developed mineral deposits in soft tissues and usually succumbed within a few weeks. More recently the ameliorative effect of magnesium and potassium on this syndrome has been described(3). Thus the guinea pig seems to be more sensitive to high levels of dietary phosphorus than the white rat and appears to have a considerably higher requirement for potassium and magnesium.

This report is concerned with one reason for the injurious effect of high phosphorus intake and the beneficial effects of potassium and magnesium.

Methods. The diets used were similar to those described by House and Hogan(3). The percentage composition of the basal ration (3884) was: acid-washed casein(4) 30 g; sucrose 47; celluflour 15; soybean oil 4; salts‡ 4. The vitamins§ added per 100 g of diet were: thiamine HCl 1 mg; riboflavin 1 mg; pyridoxine HCl 1 mg; calcium pantothenate 3 mg; niacin 5 mg; choline chloride 100 mg; inositol 100 mg; folic acid .6 mg; biotin .02 mg; vit B_{12} .003 mg; ascorbic acid 50 mg; alpha tocopherol 2 mg; 2-methyl-1, 4-naphthoquinone 1 mg; vit. A 2000 I.U. and vit D 285 I.U. In some diets gum arabic was substituted for celluflour, but since it had only a slight effect on the acid-base balance, data for gum arabic and celluflour diets were combined. The basal diet contained about 0.4% of phosphorus and 0.9% of calcium. The salt mixture of Richardson and Hogan (6) was used at a level of 5% to give a phosphorus content of 0.9% and $NaH_2PO_4 \cdot H_2O$ was added to give a phosphorus content of 1.7%. Magnesium was added as MgO, potassium and sodium as acetates, and calcium as the carbonate. All additions were made at the expense of sucrose. Diets were analyzed for calcium, magnesium, sodium, potassium and phosphorus, and are reported to the nearest tenth of a percent. Chloride and sulphur contents of diet were calculated from the composition of the salts mixture and the protein. For the purpose of calculating the acid-base balance of diets the method of Shohl and Sato(7) was employed except the results are recorded as milliequivalents of excess cations per 100 g. For growth studies guinea pigs weighing 175 to 225 g were fed *ad libitum* for 8 weeks. Most of blood and urine studies were made at the end of this period, but in the case of more severe rations, very few animals survived 8 weeks. For blood studies on these rations older animals were used and an adjustment period of 2 to 4 weeks was allowed before samples were taken. Blood for carbon dioxide capacity was taken by cardiac puncture while animals were under ether anesthesia and the determination was made on plasma by the manometric method of Van Slyke(8). Urine samples for both pH and ammonia determinations were removed directly from the bladder by catherization of anesthetized animals. The pH was determined immediately by the glass electrode and urinary ammonia determined on 5 ml of pooled sample by the method of Van Slyke and Cullen(9). Blood for determination of inorganic phosphorus was collected by clipping a clean toe nail and a 0.1 ml sample was immediately hemolyzed in water. Within 30 minutes the sample was deproteinized with

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† Present address, Ralston Purina Co., St. Louis, Mo.

‡ The salts mixture described by Hubbell *et al.*,(5) to which was added 2.5 g of $MnSO_4 \cdot 4H_2O$/100 g.

§ Folic acid was supplied by Lederle Laboratories, Pearl River, N. Y., biotin by Hoffmann-LaRoche, Nutley, N. J., and other crystalline vitamins by Merck and Co., Rahway, N. J.

TABLE I. Effect of Mineral Composition of Diet on Rate of Gain, Blood Phosphorus, Urine pH and Plasma Carbon Dioxide Capacity.

Ration No.	Variable constituents* P	Na	K	Mg	Excess cations per 100 g, mEq	Daily gain (8 wk),† g	Whole blood P, mg/100 ml	Urine pH	Plasma CO_2 cap., vol %
	%								
3884	.4	.1	.4	.1	21	3.2 (11)‡	6.0 (11)	7.3 (17)	50 (10)
3480	.9	.3	.4	.1	1	1.6 (14)	8.1 (23)	5.9 (11)	50 (3)
3475	1.7	.8	.4	.1	−20	.5 (6)	8.6 (36)	5.6 (13)	34 (10)
3479	.4	.1	1.5	.4	74	4.7 (18)	5.3 (20)	8.2 (20)	48 (14)
3515	.9	.3	1.5	.4	53	3.5 (8)	5.3 (28)	7.8 (8)	50 (6)
3477	1.7	.8	1.5	.4	32	3.8 (16)	7.5 (55)	7.2 (25)	46 (21)
16§	.9	.3	1.8	.25	—§	5.2 (24)	5.0 (58)	8.1 (11)	49 (18)

* Calcium content of all diets was 0.9% except No. 16 which contained 1.5%.
† Avg of mean daily gains of males and females.
‡ No. of animals on which avg is based is shown in parentheses.
§ Ration 16 was a stock diet composed of natural feedstuffs. Analysis was incomplete.

trichloroacetic acid and the method of King (10) was followed.

Results. The effects of different levels (0.4, 0.9 and 1.7%) of dietary phosphorus on rate of gain, blood inorganic phosphorus, urine pH and plasma carbon dioxide capacity are shown in Table I. The levels of calcium (0.9%), potassium (0.4%) and magnesium (0.1%) are those commonly used in purified diets for rats and chicks and these animals thrive on such diets even with calcium to phosphorus ratios as close as 1:1. However, in the guinea pig rate of growth was subnormal even at a low level of phosphorus (3884) unless the diet was supplemented with potassium and magnesium (3479). With higher levels of phosphorus rate of gain became progressively less until at a level of 1.7% most animals succumbed and survivors grew extremely slowly. Blood phosphorus rose to an average of 8.6 mg%, urine pH dropped to 5.6, and plasma carbon dioxide capacity dropped to 34 vol. %. These values should be compared with those obtained on animals fed the stock diet (16) composed of commonly used foodstuffs. The data show a positive correlation between rate of growth and urine pH and a negative correlation between rate of growth and blood inorganic phosphorus. It is obvious that the high phosphorus diet (3475) is an acid diet not only from the calculated deficit of cations, but also from its effect on urine pH and plasma carbon dioxide capacity. Addition of potassium and magnesium to the diets at all levels of phosphorus markedly improved growth, lowered blood phosphorus and raised urine pH. Addition of potassium and magnesium to a ration which contained 0.9% of phosphorus (3515), gave a nearly normal blood and urine picture. Addition of these cations to the excessively high phosphorus ration (3477) improved the rate of growth and raised urine pH, but did not maintain normal blood phosphorus and urine pH values. Thus, the beneficial effect of high levels of potassium and magnesium in the diet of the guinea pigs is due partly, but not wholly, to the maintenance of a positive base balance and a normal level of blood phosphorus.

Of the indices studied the urine pH seemed

TABLE II. Effect of Various Cations on the Urine pH of Guinea Pigs Fed a High Phosphorus Diet.

Ration No.	Additions Cation	%	mEq per 100 g	Urine pH	Daily gain* (8 wk)
3475	None	—	—	5.6 (13)†	.5 (6)
3895	K‡	1.1	28	6.5 (9)	.9 (10)
4120	K‡	2.2	56	7.4 (8)	2.0 (4)
3780	Na§	.6	26	6.7 (6)	1.7 (6)
3889	Na‡	.5	22	6.3 (4)	.7 (14)
3897	Na + Mg	1.0 .3	44 25	7.6 (6)	3.8 (7)
3477	K + Mg	1.1 .3	28 25	7.2 (25)	3.8 (16)
3894	Mg	.3	25	6.4 (9)	2.1 (9)
4119	Mg	.6	50	7.0 (11)	2.1 (4)
3904	Ca	1.6	80	6.8 (9)	2.0 (8)

* Avg of mean daily gains of males and females.
† No. of animals shown in parentheses.
‡ Sodium and potassium were supplied as acetate.
§ Sodium was supplied as Na_2HPO_4 which replaced $Na_2HPO_4 \cdot H_2O$ in the diet.

TABLE III. Urinary Ammonia Excretion in the Guinea Pig.

Ration No.	Excess cations per 100 g, mEq	Urinary NH_3-N, mg/100 ml	Urine pH
3884	21	1.1 (2)*	7.3 (17)
3479	74	.9 (2)	8.2 (20)
3475	−21	1.1 (3)	5.6 (13)
16	—	1.4 (3)	8.1 (11)
Human urine	—	60.0 (2)†	6.5 (2)

* No. in parenthesis indicates No. of determinations. Urine from several animals was pooled for a determination.

† These values were determined on freshly voided samples and hence do not represent a 24 hr specimen, but they are in the range commonly given for normal human urine.

to be the most sensitive to slight changes in acid-base balance. Hence, it was used to study the relative value of various cations in counteracting the injurious effects of a high phosphorus diet. Results of the growth studies and urine pH determinations are shown in Table II. Addition of each of the cations studied, potassium, sodium, magnesium and calcium, improved rate of growth and raised the urine pH. Within limits, the cations were of about equal efficacy and the effect was in proportion to the quantity added. Although potassium is more abundant than sodium in the natural diet of herbivorous animals, as a supplement to the basal diet, sodium appeared as effective as potassium either alone or in combination with magnesium. On an equivalent basis none of the cations was as effective alone as in combination. Calcium ion was quite effective in raising urine pH because in the guinea pig a relatively high proportion of the calcium is excreted by way of the kidneys (unpublished data).

The guinea pig like the rabbit(11) is more sensitive to an acid diet than most animals chiefly because it does not use ammonia to neutralize excess acids excreted by way of the kidney. In Table III are shown results of ammonia nitrogen analyses of fresh urine samples. These values on guinea pig urine agree very well with those reported for rabbit urine(12) but are of the order of 2% of the ammonia commonly found in human urine. Urine from animals fed a high phosphorus diet had a low pH but the amount of ammonia was approximately the same as that found in urine of a high pH. Thus, consumption of an acid diet caused a change from basic to acid phosphates in the urine, but it did not stimulate the excretion of urinary ammonia. Guinea pigs that consume a stock colony diet normally excrete an alkaline urine which is quite turbid. Qualitative analysis of the insoluble portion indicates that it is composed chiefly of calcium and magnesium phosphate and microscopic examination shows that phosphates are held in a noncrystalline, colloidal state. The urine excreted when animals consume a high phosphorus diet, is not turbid but is clear in appearance.

Since there is a negligible amount of ammonia excreted in the urine of the guinea pig, the shift to an acid urine appears to be the chief mechanism for conserving fixed bases. This mechanism was effective in maintaining a normal plasma carbon dioxide capacity when the level of dietary phosphorus was 0.9%, but at a level of 1.7% of phosphorus there was marked drop in carbon dioxide capacity. It seems possible that high blood phosphorus and the incidence of soft tissue calcification observed when animals consume a high phosphorus diet that is relatively low in potassium and magnesium are due in part to the inability of the guinea pig to excrete phosphorus normally under the conditions of a low base reserve.

Summary. Injurious effects of high phosphorus diets for the guinea pig, which include poor growth, metastatic calcification, and death, are due in part to the fact that this animal does not tolerate an acid diet. This has been demonstrated by the marked drop in urine pH, a decrease in plasma carbon dioxide capacity and an increase in blood inorganic phosphorus. Addition of various cations produced a more alkaline urine, but a combination of sodium or potassium and magnesium proved most beneficial. The guinea pig is highly sensitive to an acid diet because a negligible amount of ammonia is excreted by way of the kidneys.

1. Hogan, A. G., Regan, W. O., and House, W. B.,

J. Nutrition, 1950, v41, 203.

2. Hogan, A. G., House, W. B., and Regan, W. O, *Mo. Agr. Exp. Sta. Res. Bull.,* 1954, No. 567.

3. House, W. B., and Hogan, A. G., *J. Nutrition,* 1955, v55, 507.

4. McCollum, E. V., Simmonds, N., Shipley, P. G., and Parks, E. A., *Johns Hopkins Hosp. Bull.,* 1922, v33, 296.

5. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition,* 1937, v14, 273.

6. Richardson, L. R., and Hogan, A. G., *ibid.,* 1946, v32, 459.

7. Shohl, A. T., and Sato, A., *J. Biol. Chem.,* 1923, v58, 235.

8. Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry,* 12th Ed. Blakiston, Philadelphia, p651.

9. ———, *ibid.,* p828.

10. King, E. J., *Micro-Analysis in Medical Biochemistry,* Grune and Stratton, New York, 1951, p63.

11. Logan, M. A., *J. Biol. Chem.,* 1935, v109, 481.

12. Winterberg, H., *Z. Physiol. Chem.,* 1898, v25, 202.

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A Histamine-Like Component of Commercial Pitressin.* (22219)

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Several investigators have assigned an important role to pitressin as a releasing agent for ACTH. Thus, Mirsky, Stein and Paulisch(1), suggested that the antidiuretic hormone of the posterior pituitary (ADH, Vasopressin) may serve as a mechanism for ACTH release under stress. McCann and Brobeck (2), also implicated ADH as a factor for release of corticotropin. They destroyed the supraoptico-hypophyseal tract thereby inhibiting the usual output of ACTH in response to stress. However, when animals bearing hypothalamic lesions were injected with commercial pitressin, ACTH was released. Guillemin and Hearn(3), employing rat pituitaries and a tissue culture technic, demonstrated that pitressin releases ACTH from the adenohypophysis *in vitro,* whereas highly purified arginine-vasopressin obtained from du Vigneaud was without effect. The ACTH releasing activity of pitressin was attributed to an unknown contaminant probably originating in the hypothalamus. Saffran, Schally and Benfey(4), also reported that vasopressin contained a corticotropin-releasing factor as an impurity which they were able to separate from vasopressin by paper chromatography.

In view of reports(5-7) that extracts prepared from posterior pituitary powder and fresh glands contain histamine, it was considered possible that minute amounts of the amine bound to the polypeptide were carried through the extraction procedure for commercial pitressin and persisted in the finished product. To anticipate, it can be stated that commercial pitressin contains a component of unknown nature which exhibits some of the physiological characteristics of histamine and which for brevity we shall hereafter refer to as "histamine-like."

Methods. Pitressin† (Vasopressin Injection, 20 units/cc, Parke, Davis and Co.) was extracted with strong acid at boiling temperature. The material was pooled in batches containing 3200 units, in approximately 160 cc, and sufficient concentrated HCl added to make an 18% solution. The fluid was divided among 3 flasks equipped with water cooled reflux condensers and the pitressin boiled vigorously for 1 hour. The commercial material is preserved with 0.5% chlorobutanol which readily crystallizes on the condenser and was removed. After 1 hour the water condensers were replaced by reflux air con-

* Acknowledgement is made to Sharp and Dohme Division of Merck and Co., and Ciba Pharmaceutical Products Co., for support of this investigation.

† We are indebted to Dr. D. A. McGinty of Parke, Davis and Co., for 3000 units of pitressin.

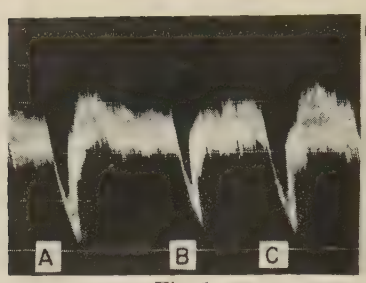

Fig. 1

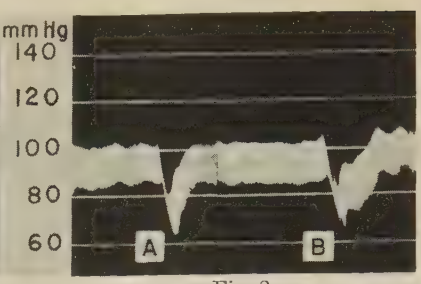

Fig. 2

FIG. 1. Cat, 2.3 kg, atropinized, etherized, carotid blood pressure. A: Pitressin extract 2 cc i.v. (625 units/kg). B: Histamine base 1.81 γ/kg i.v. C: Histamine base 3.62 γ/kg i.v. One cc of pitressin extract is equivalent to 4.17 γ/cc histamine base.

FIG. 2. Cat, 1.9 kg, atropinized, etherized, carotid blood pressure. A: Histamine base 0.3 γ/kg i.v. B: Oxytocin extract 1.5 cc i.v. (625 units/kg). Slight depressor effect. One cc of oxytocin extract is equivalent to 0.38 γ/cc histamine base compared to pitressin extract = 4.17 γ/cc of histamine base.

densers and the boiling continued for an additional 30 minutes or until the fluid was reduced to 10-15 cc. The contents of the flasks were then combined and the extract taken to dryness under reduced pressure at 40°C. The residue was washed 3 times with absolute ethanol which was removed *in vacuo*. Water was added to the desired concentration and the pH of the solution raised to that of commercial pitressin, *i.e.*, pH 3. A fine precipitate forms which is removed by centrifugation. Crude extracts prepared in the manner described retained no trace of pressor activity when tested on the blood pressure of the dog and cat. The histamine-like substance in the extracts was tested by its effect on smooth muscle of the guinea pig ileum and blood pressure of the etherized, atropinized cat. Segments of the lower ileum of fasted young guinea pigs were suspended in a 10 cc chamber containing warm, oxygenated, magnesium-free Tyrode's solution to which atropine had been added in the concentration 0.01 γ/cc. The active agent in all extracts was determined as quantitatively equivalent to histamine base and pyribenzamine was employed for inhibiting contractions of the ileum and for preventing the decline in blood pressure of the cat following extract or histamine administration.

Results. The pressor action of pitressin is abolished by boiling for 30-40 minutes in 1% HCl but a pronounced histamine-like effect is exhibited by concentrated extracts only after prolonged boiling in much stronger acid. Fig. 1 shows the effect of a concentrated extract representing 762.5 pitressin units/cc, or a 38-fold concentrate of acid-boiled commercial material. Strong depressor activity occurred which resulted in a 40 mm Hg fall in blood pressure when 2 cc were given by vein to the etherized, atropinized cat. The extract was estimated to contain activity equivalent to 4.17 γ/cc of histamine base. Oxytocin, prepared in the same manner as pitressin extract, concentrated to the same degree and administered in the same dosage to the atropinized, etherized cat, gave minor depressor effects (Fig. 2). The pitocin contained activity approximating 0.38 γ/cc of histamine base, hence possessed less than one-tenth the activity of pitressin. Apparently the histamine-like substance in the posterior lobe is chiefly confined to the pitressin fraction.

Concentrated pitressin extracts also induced typical histamine-like responses when tested on the guinea pig ileum (Fig. 3). The extracts contained 125 original pitressin units/cc, and the histamine base equivalent was approximately 0.3 γ/cc. Pyribenzamine abolished the gut response to both extract and histamine (Fig. 3 C, D).

McCann and Brobeck(2), were unable to deplete the adrenal ascorbic acid of hypophysectomized rats by intravenous injection of pitressin. We also obtained negative results in similar experiments. Extract concentrates representing 803 units/cc, and containing significant amounts of histamine-like activity in tests on the cat's blood pressure (35 mm Hg

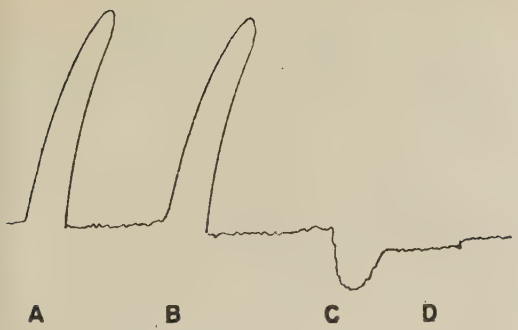

FIG. 3. Isolated ileum of guinea pig. Doses given per cc of bath fluid. A: Histamine base 0.015 γ/cc. B: Pitressin extract 0.50 cc. One cc of extract equivalent to 0.3 γ histamine base. Between B and C pyribenzamine given 0.5 γ/cc. C: Pitressin extract 0.5 cc. D: Histamine base 0.015 γ/cc.

fall), failed to produce a decline in adrenal ascorbic acid of 8 hypophysectomized rats. The extract was injected in 0.4 cc doses by vein. We are indebted to Dr. H. Megel of the Princeton Laboratories, Inc., for making the Sayers test for us.‡

Discussion. Abel and Kubota(5) reported histamine present in posterior pituitary powders and extracts. Subsequent investigators failed to detect the amine in either powders (8) or fresh glands(9). Recently, Harris *et al.*(7) were able to extract histamine from brain, hypothalamus, median eminence and both anterior and posterior pituitary tissue. The present writers have found a histamine-like substance present in extracts of acetone dried powders of the posterior lobe. Four grams of such powder when subjected to the complete extraction procedure of Code and Ing(10) for determining the presence of histamine in blood plasma and cells, and highly concentrated, contained activity approximating that of 4.19 γ/cc, of histamine base when assayed against the standard and using the guinea pig ileum and blood pressure of the cat as test objects.

‡ Recent experiments show the extract to be active in releasing ACTH from halved anterior pituitaries incubated in the Warburg apparatus using the Saffran *et al.* technic. Minute amounts of the incubation fluid given i. v. to 24 hypophysectomized rats induced a significant fall in adrenal ascorbic acid of 120 mg % in the Sayers test. Incubation fluid from the control half of the same glands gave negative results.

We were not successful in removing the active agent from pitressin or acetone dried powder by aqueous or dilute HCl extraction. It required strong acid and 90 minutes of boiling temperature to accomplish its removal. Apparently the histamine-like component of pitressin is bound to the pressor polypeptide and is released, or at any rate rendered capable of inducing physiological reactions characteristic of histamine, by some degree of acid hydrolysis. Pyribenzamine readily inhibited the response of the gut to extract and to pure histamine dihydrochloride, but it did not entirely eliminate the depressor effect of 2 cc of concentrated extract on the blood pressure of the atropinized cat. Frandsen(11) reported that the active histamine-like substance found in ultrafiltrates of dog serum differed from pure histamine since it is not destroyed by added histaminase nor attacked by histaminase of serum. The exact nature of the histamine-like component in pitressin is unknown but if it is histamine it does not appear to be present as the free amine. The writers tentatively regard it as probably histamine combined with an amino acid or peptide, but pending its isolation in pure form this is merely speculation. Certain facts seem to favor the idea and may be summarized as follows: In tests on the guinea pig ileum the behavior of the active substance in the extracts cannot be distinguished from that of histamine and like the amine its action is abolished by pyribenzamine. When highly concentrated it exerts strong depressor effects on blood pressure of atropinized, etherized dogs and cats and the slope of the pressure falls and subsequent recovery is identical with that elicited by histamine. The effect of the extract upon ileum and blood pressure can be quantitated using histamine base as the standard. Similar extracts of oxytocin which differs from pitressin by two amino acids, elicit small depressor responses in atropinized cats, but they are less than one-tenth the magnitude of those induced by pitressin. Dosage in both cases was 625 units/kg. Extracts of 4 crystalline proteins and of each of the amino acids in vasopressin also gave negative results. Investigators(2-4) have shown

that oxytocin, unlike pitressin, does not release ACTH. Histamine as the dipicrate has been recovered from posterior lobe tissue (5,6) and a histamine-like substance has been reported present in this gland and also in neighboring hypothalamic structures(7). None of these data furnish conclusive evidence that a histamine complex of some sort is the active component of pitressin extracts but they are suggestive, and it seems not unreasonable to make such an assumption as a working hypothesis.

Summary. Commercial pitressin contains minute amounts of a histamine-like component apparently bound to the polypeptide but which can be concentrated and rendered capable of inducing physiological responses characteristic of histamine when pitressin is subjected to strong acid hydrolysis and the pressor activity destroyed. Extracts of commercial oxytocin contained but one-tenth the vasodepressor activity of pitressin extracts.

1. Mirsky, I. A., Stein, N., and Paulisch, G., *Endocrinology,* 1954, v55, 28.

2. McCann, S. M., and Brobeck, J. R., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 318.

3. Guillemin, R., and Hearn, W. R., *ibid.,* 1955, v89, 365.

4. Saffran, M., Schally, A. V., and Benfey, B. G, *Endocrinology,* 1955, v57, 439.

5. Abel, J. J., and Kubota, S., *J. Pharmacol. Exp. Therap.,* 1919, v13, 243.

6. Abel, J. J., and Nagayama, T., *ibid.,* 1920, v15, 347.

7. Harris, G. W., Jacobsohn, D. J., and Kahlson, G., *Ciba Colloquia Endocrinology,* 1952, v4, 186.

8. Dudley, H. W., *J. Pharmacol. Exp. Therap.,* 1919-20, v15, 295.

9. Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.,* 1920, v43, 557.

10. Code, C. F., and Ing, H. R., *J. Physiol.,* 1937, v90, 501.

11. Frandsen, V. A., *Acta pharmak. and toxicol.,* 1948, v4, 65.

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Effects of Glucagon on Plasma Potassium.* (22220)

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Glucagon(1) has a selective action on liver in which the activity of phosphorylase is increased and this action may be the basis for increased hepatic glycogenolysis and hyperglycemia(2). The effects of glucagon mimic epinephrine action only in the liver. Glucagon has no glycogenolytic action on muscle, no cardiovascular effect(3), and no effect on hepatic vessels(4). These properties make glucagon a useful pharmacologic tool for studying the accompaniments of hepatic glycogenolysis. Our aim was to determine whether hepatic glycogenolysis was necessarily associated with increased blood potassium.

Although epinephrine hyperkalemia has been related to hepatic glycogenolysis, several facts appear inconsistent with this simple relationship. Subcutaneous epinephrine produces hyperglycemia without an associated hyperkalemia. Intravenous epinephrine produces hyperkalemia which persists for only 3 to 5 minutes(5) and hyperglycemia which continues for one-half to one hour. The relative potencies of sympathomimetic amines for the hyperkalemic effect are markedly different from their relative potencies for hyperglycemia(6,7). Dibenamine is effective in suppressing epinephrine hyperkalemia(7), but it is extremely weak as an antagonist to epinephrine hyperglycemia(8). Epinephrine hyperkalemia occurs in animals treated with phloridzin in order to deplete liver glycogen (9). Constrictor agents with weak hyperglycemic activity produce hyperkalemia(5, 10). These discrepancies were the basis for

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investigating blood potassium effects of glucagon, an agent which has hyperglycemic activity related in detail to the action of epinephrine but which has no vascular effect.

Methods. Cats, 1.85 to 3.0 kg were anesthetized with an intraperitoneal injection of 0.88 ml per kg of Dial with Urethane (each ml contains 0.1 g diallylbarbituric acid, 0.4 g urethane, and 0.4 g monoethylurea). Arterial blood samples and mean arterial pressure were obtained from a polyethylene catheter introduced into the right carotid artery and passed down into the region of the aorta. Arterial samples of approximately 3 ml were taken into heparinized syringes. Blood samples were cooled in an ice bath and the plasma separated in a refrigerated centrifuge. Control samples were taken 15 and 5 minutes before the injection. Plasma glucose was determined with the method of Nelson(11) and Somogyi(12) and plasma potassium was determined with a Beckman DU Spectrophotometer with flame attachment. Drugs were injected into a jugular or femoral vein. Glucagon‡ was an amorphous powder (Lot No. 208-158B-197) which contained approximately 50% glucagon and less than 0.05 unit of insulin per mg. In comparable experiments synthetic l-epinephrine bitartrate was used.

Results. Our data indicate that glucagon has an effect on plasma potassium similar to the effect of epinephrine. There is a marked, transient hyperkalemia followed by a slight hypokalemia of much longer duration. Glucagon induced an hyperglycemia which reached its peak much later than the hyperkalemia and hyperglycemia was present during the much longer period of hypokalemia.

The effects of glucagon (50 μg/kg i.v.) on the potassium and glucose of the arterial blood plasma are shown in Fig. 1. This is a graph of the averages of 5 experiments of similar duration. In a total of 8 experiments the average rise in plasma potassium one minute after the glucagon was administered was 2.20 mEq./l. (range 1.28-3.50) from an average control level 3.63 mEq./l. (range 3.14-4.31). The control plasma potassium and the effect of glucagon on plasma potassium were not proportional to the control plasma glucose nor to the rise in glucose. Both fasted and nonfasted cats were used. Control plasma glucose levels were from 73 to 198 mg%.

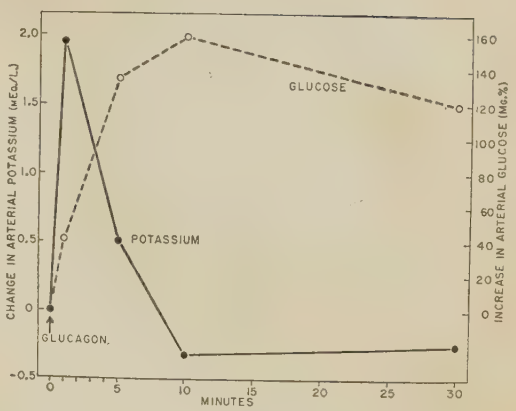

FIG. 1. Effects of glucagon (50 μg/kg) on arterial plasma potassium and glucose concentrations. Points indicate averages of 5 experiments in cats anesthetized with Dial.

Similar hyperkalemic and hyperglycemic effects were obtained with epinephrine 10 μg/kg i.v. Epinephrine produced its characteristic pressor action, whereas glucagon had no effect on the blood pressure, on the heart rate, or on the electrocardiogram. In control experiments there were no changes in arterial potassium one minute after the intravenous administration of saline or of glucagon-free insulin‡ (50 μg/kg).

With the exception of the hyperkalemic effect of glucagon, which does not appear to have been observed previously, our results confirm previous data in the literature. The subsequent hypokalemia would be expected as a consequence of the hyperglycemia which can induce a discharge of insulin and a greater tissue storage of potassium.

Discussion. It appears that glucagon, as well as epinephrine, produces a transient rise in arterial plasma potassium (presumably derived from the liver) during the activation of hepatic glycogenolysis. Since epinephrine and glucagon have the same effect on liver glycogenolysis, but only epinephrine has a vasoconstrictor action(13), it appears reason-

‡ Glucagon and glucagon-free insulin were obtained through the kind cooperation of Dr. W. R. Kirtley, Lilly Research Laboratories, Indianapolis, Ind.

able to conclude that there may be some relation of the hyperkalemic effects of these agents to hepatic glycogenolysis rather than to any vascular action. There are still several facts which do not appear to be consistent with this simple relationship. Some of these have been noted in the introduction. Although hyperkalemia and hyperglycemia appear to be associated responses, the former is completed in a few minutes while the latter persists. More related in time are the duration of hyperkalemia and the rising phase of hyperglycemia. The possibility exists that potassium loss from the liver occurs during the activation of phosphorylase, a process which occurs in less than four minutes(14), and which, under present experimental conditions, may be restored to the resting state in less than 10 minutes.

The repeated release of potassium from the liver was demonstrated by D'Silva(9) at intervals of 3 minutes. These experiments involved the repeated injection of epinephrine at 3 minute intervals and the withdrawal of arterial samples one minute after each injection. Since the liver has completed its release of potassium and may be reabsorbing potassium 3 minutes after an injection of epinephrine, D'Silva's data do not necessarily represent a continuous release of potassium.

The simultaneous loss of potassium and glycogen from the liver would appear to be in accord with Fenn's concept that there is a relation between the storage and loss of glycogen and potassium in tissues(15). However, the loss of potassium is rapid, but transient, and there is replenishment of hepatic potassium during the extended period of hyperglycemia. More information on hepatic phosphorylase activity may explain this curious situation.

Summary. In the cat, glucagon has an effect on arterial plasma potassium like that of epinephrine. It produces a transient, marked hyperkalemia followed by a much longer, but milder hypokalemia. Thus, glucagon is similar to epinephrine in that it elevates blood potassium and glucose and dissimilar in that it has no cardiovascular effects.

We are indebted to Dr. A. J. Plummer, Ciba Pharmaceutical Products, Summit, N. J., for a generous supply of Dial® with Urethane.

1. Staub, A., Sinn, L., and Behrens, O. K., *J. Biol. Chem.*, 1955, v214, 619.

2. Sutherland, E. W., and Cori, C. F., *ibid.*, 1951, v188, 531.

3. Vuylsteke, Ch., deDuve, Chr., and Nys, A., *Arch. Internat. Physiol.*, 1950, v57, 445.

4. Goldner, M. G., Jauregui, R. H., and Weisenfeld, S., *Am. J. Physiol.*, 1954, v179, 25.

5. D'Silva, J., *J. Physiol.*, 1934, v82, 393.

6. ———, *ibid.*, 1949, v108, 218.

7. O'Brien, G. S., Murphy, Q. R., Jr., and Meek, W. J., *J. Pharmacol. and Exp. Therap.*, 1953, v109, 453.

8. Harvey, S. C., Wang, C. Y., and Nickerson, M., *ibid.*, 1952, v104, 363.

9. D'Silva, J. L., *J. Physiol.*, 1937, v90, 303.

10. Houssay, B. A., Marenzi, A. D., and Gerschman, R., *Compt. rend. Soc. de Biol.*, 1937, v124, 384.

11. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

12. Somogyi, M., *ibid.*, 1945, v160, 61, 68.

13. DeDuve, Chr., *Lancet*, 1953, v2, 99.

14. Sutherland, E. W., *Ann. N. Y. Acad. Sci.*, 1951, v54, 693.

15. Fenn, W. O., *Physiol. Rev.*, 1940, v20, 377.

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Toxicity of Penicillin for the Syrian Hamster. (22221)

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Observation of the unique toxicity of penicillin to guinea pigs as compared to mice and rabbits was first made by Hamre *et al.*(1) and has since been amply confirmed(2-8). Except for generalized state of vasodilatation, very few pathological changes have been noted to account for death of the animals. The cause of this toxic effect has been variously ascribed to:—acute necrosis involving the adrenal gland, particularly of the cortex (4); allergy to penicillin(6); possible deficiency state with effects very similar to those produced by X-rays(7); and finally to change induced by penicillin in predominantly Gram positive intestinal flora of guinea pigs thereby permitting overgrowth of non-sensitive Gram negative bacteria and production and resorption of toxins of the latter(8).

Existence of this unusual sensitivity to penicillin has not been reported in other animals. During experiments on Syrian hamsters treated with penicillin, it was first noticed that mortality rate of treated group consistently exceeded that of untreated controls. Because of observations in guinea pigs and of their widespread use in research, we investigated the possible toxic effect of penicillin upon hamsters.

Methods. Syrian hamsters, of either sex, weighing 50-60 g were employed in groups of 10. A single injection of crystalline penicillin G potassium (Bristol) was administered to each hamster in dose and route indicated in Table I. Volume injected subcutaneously was 0.25 ml and intraperitoneally 1 ml. All animals were kept on normal diet and observed 21 days. Symptoms were noted, total mortality and days of death were recorded for each group. Because of reported resistance of mice and susceptibility of guinea pigs to penicillin, these were included for comparison. Two groups of 10 Swiss mice, weighing 15-20 g and 2 groups of 10 guinea pigs, weighing approximately 200 g received 100000 units of crystalline penicillin G potassium. One group of each species was injected subcutaneously and the other intraperitoneally in 0.25 ml and 1 ml amounts, respectively. In addition, groups of each species received the same volume of physiological saline by same route as those receiving penicillin.

TABLE I. Toxicity to Syrian Hamster and Mortality Time of One Injection of Variable Doses of Crystalline Penicillin G Potassium Administered Subcutaneously and Intraperitoneally.

	Subcutaneous		Intraperitoneal	
Penicillin (units)	Mortality	Days of death (No.–day)	Mortality	Days of death (No.–day)
×1000				
1	1/10*	1–7	0/10	—
5	"	1–3	2/10	1–2; 1–3
10	3/10	1–3; 2–4	4/10	2–2; 2–3
25	"	3–3	5/10	3–3; 2–4
50	5/10	1–3; 2–4; 1–8; 1–12	9/10	3–3; 4–4; 1–5; 1–6
100	9/10	4–4; 4–5; 1–7	10/10	3–4; 6–5; 1–6

* Numerator = No. died. Denominator = Total No. animals injected.

Results. No deaths were observed in control animals in the 3 animal species under consideration that received physiological saline either subcutaneously or intraperitoneally. From Table I, it may be noted that a single injection of penicillin starting with 1000 units subcutaneously and 5000 units intraperitoneally is toxic for some hamsters. Increasing the dose by either route results in progressively higher mortality until a 90-100% rate is observed with 100000 units. Intraperitoneal administration proved more toxic than did subcutaneous, as evidenced by consistently higher mortality rate in animals receiving the antibiotic by former route. The peak mortality period for varying doses of penicillin is also illustrated in Table I. No deaths occur on 1st day, only 3 out of 52 deaths are found on 2nd day and vast majority of deaths takes place on 3rd, 4th and 5th days following injection. Few deaths supervene after this time. It is of interest to

TABLE II. Comparative Susceptibility of Swiss Mice, Guinea Pigs and Syrian Hamsters to One Injection of 100000 Units of Crystalline Penicillin G Potassium.

	Subcutaneous		Intraperitoneal	
Animal	Mortality	Days of death (No.–day)	Mortality	Days of death (No.–day)
Mice	0/10	—	0/10	—
Guinea pigs	10/10	1–3; 3–4; 3–5; 2–6; 1–7	8/10	1–3; 2–4; 4–6; 1–7
Hamsters	9/10	4–4; 4–5; 1–7	10/10	3–4; 6–5; 1–6

note that increased dosage of penicillin produces higher mortality rate but no shortening of survival time. On the contrary, with higher doses, peak mortality is delayed a day or two rather than accelerated as compared with lower amounts. Thus peak mortality day with 25000 units or less is the 3rd, with 50000 units, the 4th, and with 100000 units the 5th day. The picture presented by the hamsters was similar to that previously described for guinea pigs. Food and water intake was diminished, hair became ruffled, animals were lethargic and huddled together in a shrunken posture.

By contrast, as seen in Table II, Swiss mice weighing only 15-20 g readily tolerate a single injection of 100000 units of penicillin administered either subcutaneously or intraperitoneally. They remain alive and active throughout the 21-day observation period. On the other hand, the same dose of antibiotic causes death in 80-100% of guinea pigs and in 90-100% of hamsters similarly treated, depending upon route of administration. The mortality pattern in hamsters closely parallels that in guinea pigs as toxicity of penicillin for both is of the delayed type. Thus in both species no deaths are noted before the 3rd day or after 7th day following injection.

In the light of our findings, reevaluation of experimental data concerned with Syrian hamsters either treated with penicillin or injected with material sterilized with penicillin would seem to be indicated. Although amounts less than that reported in this study as capable of producing toxic effects in hamsters might have been employed, the cumulative effect of multiple small injections might well produce the same result and render observed experimental data inaccurate.

Summary. A single injection of crystalline penicillin G potassium administered subcutaneously or intraperitoneally is toxic for hamsters in proportion to amount administered. Toxicity is of the delayed type, the vast majority of deaths occurring on 3rd, 4th and 5th days following injection. This parallels the reported experience with guinea pigs and is in contrast to Swiss mice who easily tolerate very large doses of this antibiotic. In the light of these findings, reevaluation of experimental data concerned with Syrian hamsters either treated with or injected with material sterilized with penicillin would seem to be indicated.

1. Hamre, D. M., Rake, G., McKee, C. M., and Macphillamy, H. B., *A. J. Med. Sci.,* 1943, v206, 642.

2. Heilman, F. R., and Herrell, W. E., *Proc. Staff Meet. Mayo Clinic,* 1944, v19, 89.

3. Rake, G., and Richardson, A. P., *Ann. N. Y. Acad. Sci.,* 1946, v48, 143.

4. Cormia, F. E., Lewis, G. M., and Hopper, M. E., *J. Inv. Derm.,* 1947, v9, 261.

5. Stuart, P., and Slavin, G., *Nature,* 1951, v167, 319.

6. Ambrus, C. M., Sideri, C. N., Johnson, G. C., and Harrisson, J. W. E., *Ant. and Chem.,* 1952, v2, 521.

7. Stevens, K. M., and Gray, I., *ibid.,* 1953, v3, 731.

8. De Somer, Pa., Van DeVoorde, H., Eyssen, H., and Van Dijck, P., *ibid.,* 1955, v5, 463.

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A Serum Peptidase and Its Possible Role in Protease Inhibition. (22222)

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The presence of a number of peptidases in serum has been detected by the use of synthetic substrates such as leucyl-glycyl-glycine, glycyl-glycyl-glycine, glycyl-leucine, etc. The function of serum peptidases, however, is still largely unknown. The method described by Gomori(1,2), using a chromogenic substrate, is sensitive and simple enough to lend itself easily to physiological and clinical studies. The present paper reports the results of investigations carried out with Gomori's method. These results point to the possibility that part of the well-known antiproteolytic activity of serum is due to enzymatic destruction of proteases.

Methods. Determination of enzyme activity is based on the color reaction between beta-naphthylamine and certain diazotized dyes. When glycyl-beta-naphthylamine is hydrolyzed, the amine set free gives a purple colored compound in combination with the dye. Intensity of color, measured spectrophotometrically, indicates degree of hydrolysis of substrate. *Reagents.* 1. Glycyl-beta-naphthylamine (GBNA) was prepared according to Gomori(2) and used at concentration of 0.0002 M (sample used was prepared and kindly supplied by A. J. Merritt, U. S. Vitamin Corp.). 2. Veronal buffer, pH 7.4, 0.15 M in physiological saline. 3. Acetate buffer, pH 4.2, 1 M, containing 10% Tween 20. 4. Red B Salt (Matheson, Coleman and Bell) in 0.25% solution, to be prepared shortly before use. *Procedure.* The enzyme solution is made up to 6 ml with veronal buffer and placed in water bath at 37.5°C. The reaction is started by addition of 2 ml of GBNA solution. After required interval, usually 30 min., the reaction is stopped by adding 1 ml of Tween-acetate buffer solution. The purpose of Tween 20 is to solubilize the colored compound formed when 1 ml of dye is added to the reaction mixture. Color develops rapidly and remains stable for over an hour. Test solutions are read in Coleman Universal spectrophotometer at 550 mμ against blank containing all reagents except the enzyme. *Color reaction* has a high sensitivity and may be made even more sensitive by reducing volume of reagents. The beta-naphthylamine-dye compound has a molar extinction coefficient of 43800 at 550 mμ. With this procedure 2.5% hydrolysis can still be measured (0.01 μM in final volume of 10 ml). Results of experiments are expressed in terms of μM of substrate hydrolyzed. *Inhibition of protease* by serum was measured by means of method described previously(3). Blood samples were collected by venous or heart puncture and allowed to clot at 37.5°C. Unless otherwise stated, the serum was used shortly after separation. When kept for longer periods, serum was frozen to –20°C. Tissue extracts were prepared according to method described by Fruton(4) and extracts were kept frozen.

Results. Serum samples from laboratory animals and healthy human subjects were tested for peptidase activity on GBNA. Table I shows mean values of GBNA hydrolysis by sera from different species at stated concentrations. Although enzyme activity varied from one species to another it was remarkably constant within the same species.

Kinetic studies were carried out with guinea pig serum because of its high activity. They showed that rate of GBNA hydrolysis decreased as the reaction proceeded (Fig. 1).

TABLE I. Hydrolysis of GBNA by Sera from Different Species.

Species	Conc., ml	μM hydrolyzed in 30 min.	± S.E.	No.
Guinea pig	.1	.089	.004	24
Human	.3	.024	.005	4
Mouse	.3	.020	.005	3*
Rabbit	.5	.062	.008	6
Dog	.5	.032	.005	4
Rat	.5	.028	.002	12

* 3 pooled samples from 4 mice each.

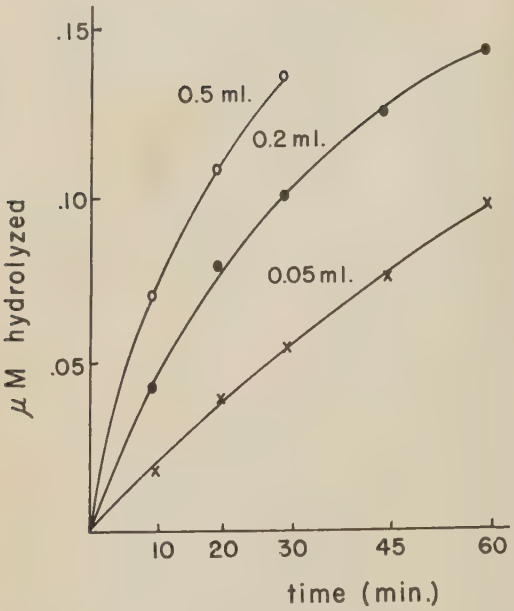

FIG. 1. Hydrolysis of GBNA by guinea pig serum. Abscissa: duration of the reaction (min.); ordinate: μM of substrate hydrolyzed by the amounts of serum indicated.

It was found also, as shown in Fig. 2, that hydrolytic activity was not linearly related to enzyme concentration but approximated more closely log [E]. Rate of hydrolysis, however, was proportional to substrate concentration within limits of experiment. The reaction is not inhibited by its end-products: neither glycine nor beta-naphthylamine have any inhibitory action on the enzyme at the concentrations at which they are released during the reaction.

Hydrolysis of GBNA by guinea-pig serum was inhibited by cyanide and ethylenediamine-tetra-acetic acid (EDTA). This suggested that the enzyme action requires some metallic ion. None of the ions tried (Ca, Mg, Mn, Co, Ni, Cu, Zn), however, activated hydrolysis or reversed action of the chelating agent. Most of the metals tried inhibited GBNA hydrolysis, some of them (Cu, Zn) at very low concentrations.

Enzyme action was also inhibited by a number of amines (methylamine, tryptamine, epinephrine, norepinephrine, histamine, 5-hydroxy-tryptamine). The amino-acids corresponding to some of them (histidine, tryptophan) were inactive. Ammonium salts can also inhibit peptidase; quaternary ammonium compounds (choline, acetylcholine), however, had no inhibitory action. The basic protein, protamine, had a comparatively strong inhibitory action; on molar basis approximately 10^{-7} would be required for 50% inhibition.

The GBNA-hydrolyzing enzyme was found in the albumin fraction when serum was diluted 20-fold with distilled water and precipitated at pH 5.4. Heating serum to 56°C for 30 minutes did not significantly decrease the enzyme activity. Dialysis in the cold for 48 hours against saline did not cause loss of activity. Serum which was kept frozen for 24 hours showed consistently higher activity (by 15 to 25%) than fresh serum.

A few experiments with tissue extracts showed that guinea-pig lung and skin were able to hydrolyze GBNA (0.130 μM by 100 mg of skin) while rat tissues were inactive. Human urine showed slight activity (0.016 μM/ml). It appeared that the GBNA-hydrolyzing enzyme had striking similarities with the substances variously designated as antitrypsin, antifibrinolysin, antiplasmin, protease inhibitor, etc. GBNA hydrolysis and plasmin (fibrinolysin) inhibition follow similar reaction kinetics: the rate is highest in the

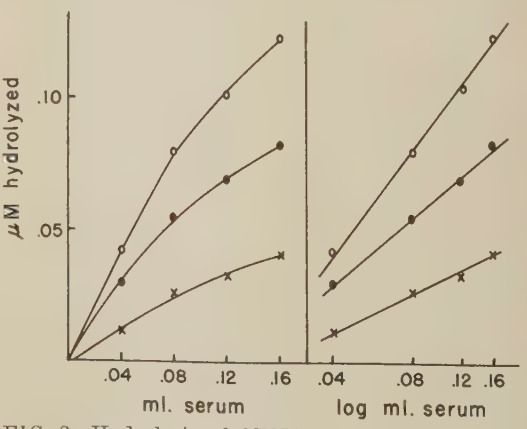

FIG. 2. Hydrolysis of GBNA as a function of enzyme concentration. Ordinate: μM of substrate hydrolyzed in 30 min. at the substrate concentration indicated; abscissa: amount of guinea pig serum added to the substrate (ml) at arithmetic (left) and logarithmic (right) scale.

× = .2 μM GBNA
● = .4 " "
○ = .6 " "

TABLE II. Inhibition of GBNA Hydrolysis by Fibrinolysin and Trypsin.

Inhibitor,* mg		μM hydrolyzed in 30 min. by .1 ml guinea pig serum	.2 ml guinea pig serum
	0	.085	.110
Plasmin	10	.078	.096
	25	.060	.076
	50	.040	.045
Trypsin	0.4	.082	.104
	1.0	.061	.080
	2.0	.037	.046

* Conc. for total volume of 8 ml.
Serum and inhibitor were left in contact for 10 min. before addition of substrate.

first 5 min. and increases linearly with the log of serum concentration(3,5). Both reactions are inhibited by EDTA and inhibition is not reversed by addition of Ca(6). Both systems are inhibited by low concentrations of Cu and protamine(7), by certain amines and ammonia(8). On isoelectric precipitation, both properties of serum are found in the supernatant(9).

One more evidence is supplied by the observation that trypsin (Tryptar, Armour Laboratories) and plasmin (bovine preparation kindly supplied by Dr. E. C. Loomis, Parke, Davis and Co.) inhibit hydrolysis of GBNA by guinea pig serum (Table II). Several other globulin fractions from guinea pig, rabbit and human serum showed no inhibitory action. Plasminogen did not inhibit but inhibitory activity appeared after activation with streptokinase. Kinetic studies of this inhibition are now in progress; they indicate that it is competitive and reversible. Reversibility of the inhibition was substantiated by the following experiment. Guinea pig serum was incubated with plasmin for 30 min. The mixture was then diluted with distilled water and the globulin fraction precipitated out at pH 5.4. Plasmin was in the precipitate while GBNA-peptidase remained in solution. By testing the 2 fractions, it was seen that plasmin was partly destroyed but the peptidase was completely recovered. GBNA and proteases seem to be competing substrates for the same enzyme.

The GBNA-hydrolyzing enzyme, however, is not the only protease-inhibiting factor in serum. There are probably two(10) and possibly three(8) antiproteases in human serum. Table III shows the correlation between plasmin inhibition and GBNA hydrolysis by sera from different species. It is seen that in guinea pigs and possibly rabbits the plasmin-inhibiting action of serum may be due to GBNA-peptidase. In other species, particularly in man, other inhibitors may play a more important role. Bovine serum has no action on GBNA and purified bovine antiplasmin prepared by Loomis(11) also fails to act on this substrate.

The plasmin inhibitor of guinea-pig serum controlled by pituitary and adreno-cortical hormones(3,12) may be identical with GBNA-peptidase. Table III shows that sera from guinea pigs treated with cortisone (5 mg/kg, 4 hours before bleeding) had an increased action on GBNA hydrolysis and plasmin inhibition. The opposite was observed in sera of animals treated for 4 days with 5 mg/kg of pituitary growth hormone (obtained by courtesy of Armour Laboratories).

Discussion. It is uncertain whether the enzyme detected by its action on GBNA is identical or not with one of the peptidases already described in serum. It seems to be closest to the enzyme hydrolyzing glycyl-leucine which, however, is activated by Zn(13). The relationship of GBNA peptidase with metallic ions remains unknown; its activation by Co, observed in human serum(2), was not confirmed in guinea pig serum. It seems that, if it requires any ion at all,—as is suggested by EDTA inhibition,—this has to be firmly

TABLE III. GBNA Hydrolysis and Plasmin Inhibition by Sera from Different Species.

Species	μM GBNA hydrolyzed/ml in 30 min.	Units plasmin inhibited/ml
Mouse	.030	40
Rat	.036	44*
Human	.038	51
Dog	.041	47
Rabbit	.081	36
Normal guinea pig	.143	66*
Cortisone-treated guinea pig	.194	85*
Growth hormone-treated guinea pig	.082	31*

* Results published in a previous study(3).

bound to the protein since it cannot be removed by dialysis.

Inhibitory action of certain biologically important amines (serotonin, histamine) is unlikely to have any physiological significance because they can hardly occur at the required concentrations.

On the basis of present data it cannot be decided whether GBNA-hydrolyzing enzyme is a true peptidase or a proteinase whose specificity is satisfied by the structure of the synthetic substrate. To inactivate trypsin or plasmin, it has to hydrolyze off their active groups. Since the nature and length of these groups is unknown we cannot even speculate on site of action of the enzyme.

The fibrinolytic system and other similar systems present in tissues appear to play a role in certain physiological and pathological processes(14). Rate of protease inhibition is an important factor controlling extent and duration of the processes. If the interpretation of present results is confirmed, they may supply an interesting example of one enzyme controlling the activity of another.

Summary. A peptidase, detectable by its action on chromogenic substrate glycyl-beta-naphthylamine, is present in serum of most species. In guinea pig serum, hydrolysis of GBNA and protease inhibition show a marked parallelism: they have a similar kinetic behavior, they are inhibited by the same agents and are under the influence of the same hormones. Competitive inhibition of peptidase action by plasmin and trypsin suggest that proteases are substrates for peptidase and that antiproteolytic activity of guinea pig serum, is due, partly at least, to enzymatic destruction of proteases. In some other species this mechanism may be less important.

1. Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 570.

2. ———, *ibid.,* 1954, v87, 559.

3. Ungar, G., Damgaard, E., and Hummel, F. P, *Endocrinology,* 1951, v49, 805.

4. Fruton, J. S., *J. Biol. Chem.,* 1946, v166, 721.

5. Guest, M. M., Daly, B. M. Ware, A. G., and Seegers, W. H., *J. Clin. Inv.,* 1948, v27, 785.

6. Burdon, K. L., and Guthrie, R. K., *USAF School of Aviation,* 1953, Proj. No. 21-3501-0004. Rep. No. 2.

7. Kaulla, K.N.v., *Sang,* 1951, v22, 720.

8. Ratnoff, O. D., Lepow, I. H., and Pillemer, L., *Bull. Johns Hopkins Hosp.,* 1954, v94, 169.

9. Grob, D., *J. Gen. Physiol.,* 1942, v26, 405.

10. Shulman, N. R., *J. Exp. Med.,* 1952, v95, 571 and 593.

11. Loomis, E. C., Ryder, A., and George, C. J., *Arch. Biochem.,* 1949, v20, 444.

12. Gray, E. J., Volkringer, E. T., Chamovitz, D. L., Kocholaty, W. F., and Jensen, H., *Endocrinology,* 1953, v52, 228.

13. Smith, E. L., *Adv. in Enzymol.,* 1951, v12, 191.

14. Ungar, G., *Lancet,* 1952, v2, 742. *Internat. Arch. Allergy,* 1953, v4, 258.

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Observations on a Precipitin Reaction Between Serum of Patients with Rheumatoid Arthritis and a Preparation (Cohn Fraction II) of Human Gamma Globulin.* (22223)

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Since the observation by Rose *et al.*(1) of agglutination, by sera of many patients with rheumatoid arthritis, of sheep red cells sensitized with nonagglutinating quantities of rabbit antisheep cell serum (amboceptor), a number of modifications of this test system have been described. These fall into two types: those using sheep red cells sensitized with amboceptor against patient's serum or its euglobulin fraction and a system described by Heller in 1954(2). In the latter, sheep red cells, previously treated with tannic acid, are coated with pooled human gamma globulin (Cohn Fraction II). These treated cells are agglutinated when exposed to the serum of patients with rheumatoid arthritis.

In numerous clinical studies(3,4), these test systems have shown a high degree of specificity in patients with indisputable evidence of rheumatoid arthritis. That these tests remain positive when the overt inflammatory reactions have been suppressed by steroid therapy has suggested to us that the circulating factor or factors (rheumatoid factor, R.F.) responsible for these serological reactions may be closely concerned with the mechanisms of this disease. While studying the mechanisms involved in the Heller FII test, it appeared likely that there existed a specificity of interaction between some substance in human pooled gamma globulin and the rheumatoid factor (R.F.), the interaction product of these two on sheep red cells producing visible agglutination. Although the R.F. in the patient's serum has been characterized as to its localization in the Cohn ethanol fractionation system(5), its further identification has been handicapped by the need for a sheep cell system to detect its presence. The following study was undertaken to eliminate the red cell from this system and to precipitate specifically the R.F., thus making it amenable to chemical and immunochemical study.

Methods. Lyophilized pooled human gamma globulin obtained from the American Red Cross dissolved in pH 8 buffered saline to 2.5 g % concentration was absorbed free of naturally occurring antibodies to sheep red cells and heated to 56°C for 30 minutes to inactivate complement. This material was used for coating the sheep red cells for the conventional Heller test and for the precipitation studies. It has been demonstrated previously that addition of extremely small quantities of this gamma globulin to a positively reacting serum from a patient with rheumatoid arthritis results in a negative FII test presumably by the interaction of the gamma globulin added with the R.F. of the patient's serum(2). The sera of subjects studied were complement inactivated and absorbed free of naturally occurring sheep cell antibodies. This treatment of the sera allowed both the precipitin test and the FII test to be done on the same specimen.§ Nitrogen content of gamma globulin solutions was determined by the Kjeldahl method. Euglobulin was prepared by adding equal volumes of serum and distilled water in cellophane bag and dialyzing against distilled water for 48 hours at 4°C. The insoluble fraction (euglobulin) was washed with distilled water until washings were protein-free (Biuret), and redissolved in saline to serum concentration. Initial studies showed that when FII positive serum or its euglobulin fraction was

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† Fellow, Arthritis and Rheumatism Foundation.

‡ Visiting Fellow in Medicine, supported by grant from Westminster Hospital, London, England.

§ Precipitation occurs, however, even if the complement of serum and gamma globulin solution is not inactivated.

mixed with varying amounts of gamma globulin, a precipitate resulted. This precipitate formed immediately at the interface of the two components with the larger amounts of gamma globulin when an overlay technic was used. In all studies of precipitate formation, the tubes were first observed for precipitate at the interface, then mixed, allowed to stand at room temperature for 1 hour, placed at 4°C, and finally read by observing the precipitate while shaking the tubes. Aliquots of the supernatants were tested for excess of gamma globulin by the addition of the same serum, for excess rheumatoid factor by the FII test and by the addition of gamma globulin.

Results. Table I shows the result of combining a constant amount of an FII positive serum (0.5 cc of 1:7 dilution in pH 8.0 buffered saline) with varying amounts of gamma globulin. The plus sign indicates the presence of an opalescent gelatinous precipitate. Although quantitative immunochemical studies are not completed, this table suggests an equivalence zone with gamma globulin excess in the supernatant on one side and rheumatoid factor on the other(6).

Table II shows the result of simultaneous dilution of a serum euglobulin and of gamma globulin. Interpreted in the manner of a single antigen-antibody precipitin system, the rapid diminution in the amount of precipitate

TABLE I. Precipitation Reaction of Gamma Globulin with Rheumatoid Serum. Serum #145. 0.5 cc of 1:7 buffered saline dilution. FII titer > 56,000. 4°C for 4 days.

mg γ glob. N. in 0.5 cc	Degree of ppt. after 4 days at 4°C	FII test on supernatant	Excess γ globulin in supernatant	Excess R factor in supernatant
.440	4+	0	+	0
.408	"	0	+	0
.377	"	0	+	0
.346	"	0	+	0
.314	"	0	+	0
.283	"	0	+	0
.251	"	0	+	0
.220	"	0	+	0
.188	"	0	+	0
.157	"	0	+	0
.126	"	0	faint pos.	0
.0943	"	0	*Idem*	faint pos.
.0628	"	0	0	*Idem*
.0314	3+	896	0	"

TABLE II. Simultaneous Dilution of Gamma Globulin and Rheumatoid Serum Euglobulin. Dilution of serum euglobulin from a patient with rheumatoid arthritis (FII titer > 56,000) in buffered saline pH 8.0. 4°C for 48 hr. Precipitate measured on a scale of 0 to 4 plus.

mg γ glob. N. in 0.3 cc	Undil.	1:2	1:4	1:8	1:16	1:32	Buffered saline
.381	4+	3+	3+	2+	1+	0	0
.255	"	"	2+	"	"	±	0
.192	"	"	3+	"	2+	1+	0
.096	"	"	2+	"	1+	"	0
.048	3+	2+	"	1+	"	"	0
.0192	2+	"	"	"	"	±	0
.0096	1+	1+	1+	"	"	"	0
.0048	"	"	"	"	"	0	0
.00192	"	"	0	0	0	0	0
.00096	"	0	0	0	0	0	0
Buffered saline	±	0	0	0	0	0	0

formed as the euglobulin was diluted (no precipitate at dilution 1:32), while precipitation still occurred with 200-fold dilution of gamma globulin, suggests that the latter or some component of it behaves as the antigen in this system(6).

The sera of 18 of 21 individuals with the clinical diagnosis of rheumatoid arthritis and a positive FII test showed precipitation under these conditions; no precipitation occurred with the other three. Of 27 individuals with a variety of joint diseases but with negative FII tests, none showed precipitation.

Summary. The precipitin reaction which has been observed will permit study of the 2 factors, one found in serum of patients with rheumatoid arthritis, the other in human gamma globulin, which interact to give a positive FII test (Heller). This reaction eliminates the need for sheep cells and lends itself to investigation by technics of quantitative immunochemistry(6).

1. Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 1.

2. Heller, G., Jacobson, A. S., Kolodny, M. H., and Kammerer, W. H., *J. Immunol.*, 1954, v72, 66.

3. Bunim, J. J., Sokoloff, L., Williams, R. R., and Black, R. L., *J. Chronic Diseases,* 1955, v1, 168.

4. Jacobson, A. S., Kammerer, W. H., Wolf, J., Epstein, W., and Heller, G., *Amer. J. Med.,* in press, 1956.

5. Heller, G., Kolodny, M. H., Lepow, I. H.,

Jacobson, A. S., Rivera, M. E., and Marks, G. H., *J. Immunol.*, 1955, v74, 340.

6. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry,* Charles C. Thomas, Springfield, Illinois, 1948.

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Growth of Human Epidermoid Carcinomas (Strains KB and HeLa) in Hamsters from Tissue Culture Inocula.* (22224)

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Eagle has reported the isolation of a human epidermoid carcinoma (strain KB) directly from a biopsy specimen(1) in a new medium which satisfies the specific amino acid and vitamin requirements of a mouse fibroblast (strain L) and the HeLa strain of epidermoid carcinoma(2-5). Since strain KB was isolated directly in these culture media without the intervening use of experimental animals, the purpose of the present report is to record its successful transplant from *in vitro* cultures to the cheek pouches of Syrian hamsters. Similar experiments with the HeLa cell also were undertaken, since this tissue culture strain apparently has not yet been studied in the hamster cheek pouch.

Methods. Strain KB, isolated Dec. 21, 1954 and maintained by serial subculture in these new media(1), and strain HeLa(6), similarly maintained in these media for more than a year, were obtained through the courtesy of Dr. Harry Eagle, National Institutes of Health. Both strains had been cultivated in these laboratories for some 4-6 months prior to the present experiments by regular subculture in the medium described by Eagle (2-5), which contains 13 essential amino acids, 7 essential vitamins, glucose and salts, each in the concentration necessary for maximal growth of these 2 cell lines(1-5). The essential serum protein was provided in the present experiments by 10% whole, fresh human serum. The layer of cells growing on the surface of the culture flasks was removed for subculture and for animal experiments by replacing the culture fluid in 8-10-day-old cultures with fresh media containing Difco "1:250" trypsin in a final concentration of 0.125%, and incubating the flask *circa* 5 min at 37°C. The dispersed cells were then sedimented at 5-600 rpm, washed 2 or 3 times with 10-15 ml of culture media to remove the trypsin and resuspended in a convenient volume of complete culture media. The suspensions were shaken gently to disperse the cells, and after enumeration by direct haemocytometer counts, diluted so that the desired number of cells was contained in a volume of 0.1 ml of media. Syrian hamsters, 60-80 g in weight, were prepared for implantation as described by Lutz *et al.*(7). The 0.1 ml of culture media containing the desired inoculum was implanted under the epithelium of the cheek pouch with a 24 gauge needle. Cortisone acetate in subcutaneous doses of 2-3 mg was administered simultaneously with implantation and twice weekly post-implantation(8).

Results. Strain KB. The implantation of 16,000 cells produced a visible nodule in the cheek pouch (Fig. 1a) following a latent period of 56 days. The nodules continued to grow and eventually filled the pouch, presenting the gross appearance of a typical cheek pouch tumor (Fig. 1b). The histology of these tumors† is consistent with that of an

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† We wish to thank Dr. J. Bernstein, Department of Pathology, Children's Medical Center, for histological study of these tumors.

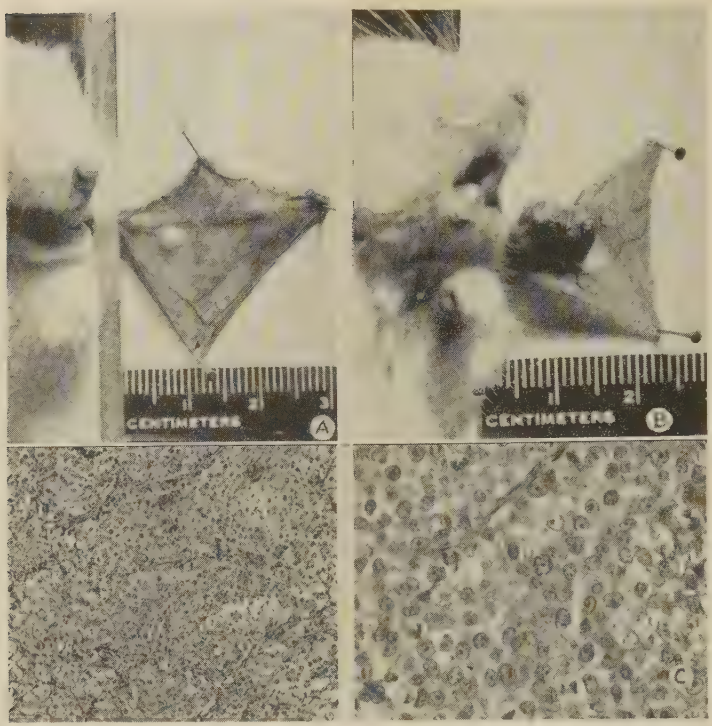

FIG. 1. Implantation of human epidermoid carcinoma, strain KB in hamster cheek pouch from tissue culture inocula. a. Implantation of 16,000 cells, 56 days. b. Same tumor, 76 days. c. Histologic section, 76 days. H. & E., 140 × and 440 ×.

epidermoid carcinoma (Fig. 1c). The latent period following implantation from *in vitro* culture has decreased progressively with successive hamster passages until now after the 12th passage, it must be transplanted every 10 days. The tumor grows in about 80% of the animals implanted, and if not transplanted on schedule, ulceration develops and the tumor becomes necrotic between 12-15 days. The animals then expire, usually as the result of secondary infection. Preliminary dose-response experiments done in duplicate with bilateral implantation in each animal indicate that the implantation of *circa* 15-20 cells results in the development of a tumor following a latent period of 153 days. Samples of tumor from the 9th passage of strain KB in cortisonized hamsters were implanted successfully in the cheek pouches of untreated animals. This tumor line now has been transplanted through 3 successive passages in non-conditioned hamsters. The tumor growth rate thus far is slower than in cortisone-treated animals. These tumors if not transplanted, undergo ulceration and necrosis between 22 and 25 days.

Tissue culture inocula of strain KB also have been implanted subcutaneously into LAF_1 mice bearing intramuscular transplants of the homologous ACTH secreting pituitary tumor reported by Furth *et al.*(9) according to the method described by Handler(10). Tumors developed in 80% of the mice bearing the pituitary tumor, attaining volumes of approximately 1 ml in thirty days. All mice died of the effects of the pituitary tumor. Thus far, strain KB has failed to grow in normal mice.

Strain HeLa. The implantation of 18,600 cells produced a nodule in the cheek pouch (Fig. 2a) following a latent period of 14 days. These nodules developed the gross appearance of typical cheek pouch tumors (Fig. 2b), the histology† of which is consistent with that of an epidermoid carcinoma (Fig. 2c). The latent period following implantation from *in vitro* culture has decreased to 10 days after 4 successive hamster passages. The tumor

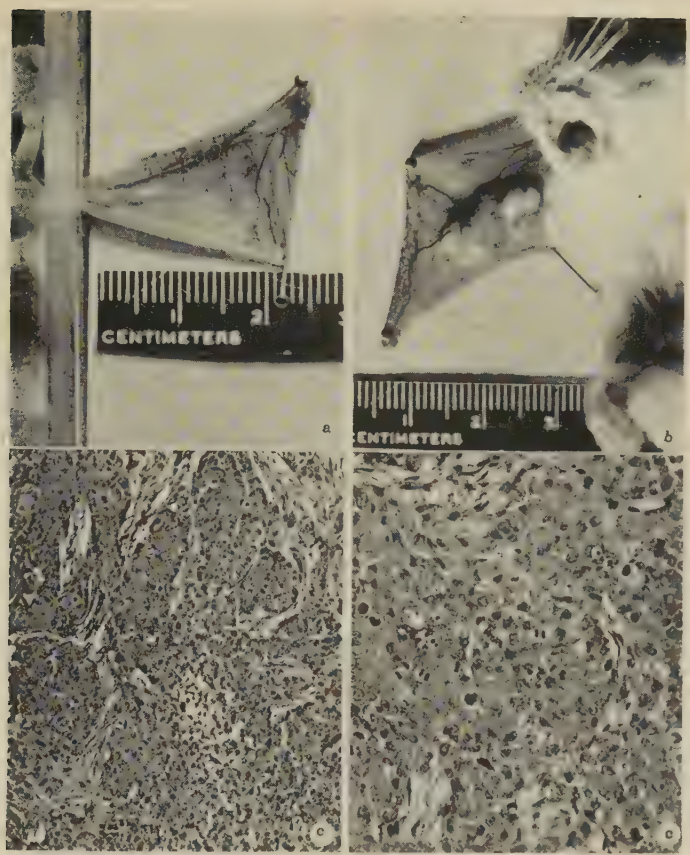

FIG. 2. Implantation of human epidermoid carcinoma, strain HeLa in hamster cheek pouch from tissue culture inocula. a. Implantation of 18,600 cells, 14 days. b. Same tumor, 34 days. c. Histologic section, 34 days. H. & E., 75 × and 220 ×.

grows in 60% of the animals implanted, and unless transplanted every 18 days, the cheek pouch ulcerates, the tumor becomes necrotic between 20-24 days and the animals die of secondary infection. Preliminary dose-response experiments done in duplicate with bilateral implantation in each animal indicate that the implantation of *circa* 15-20 cells results in the development of a tumor following a latent period of 92 days.

Discussion. The present experiments with strain KB represent the first instance in which a human tumor isolated directly from a biopsy specimen in these new media has been transplanted from cultures to experimental animals. Similarly, so far as is known, strain HeLa has not heretofore been implanted successfully in the hamster cheek pouch. These observations indicate a simple method by which various tumor cell lines isolated and/or maintained in these media can be checked periodically for tumor production *in vivo,* and further suggest the possibility that the same tumor cell lines may be established and used in parallel in these media and in the hamster cheek pouch for the comparative screening of potential chemotherapeutic agents.

The uniform tumors produced by measured tissue culture inocula should be particularly useful in the chemotherapeutic study of cheek pouch tumors. Quantitated cell suspensions prepared by the trypsinization of biopsy specimens(11) of certain other human tumors being carried in hamsters(12) have been equally effective when implanted into the hamster cheek pouch, as have been similar hamster and mouse tumor inocula when im-

planted appropriately in homologous hosts (12). These preliminary experiments also suggest that the latent period may be predetermined within reasonable limits by the number of tumor cells contained in the inoculum.

Summary. 1. The KB and HeLa strains of human epidermoid carcinoma have been established in the cheek pouches of golden hamsters by means of quantitated suspensions prepared from tissue cultures in a medium providing the specific amino acid and vitamin requirements of these cell lines. 2. Strain KB, isolated directly from a biopsy specimen in these media, has been grown in LAF_1 mice bearing homologous ACTH secreting pituitary tumors. This strain also has been established in the cheek pouch of non-conditioned hamsters but thus far has failed to grow in non-conditioned mice. 3. The use of quantitated tissue culture inocula for the production of tumors in the hamster cheek pouch for chemotherapeutic studies is suggested.

1. Eagle, H., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 362.

2. ———, *ibid.*, 1955, v89, 96.

3. ———, *J. Biol. Chem.*, 1955, v214, 839.

4. ———, *J. Exp. Med.*, 1955, v102, 1955.

5. ———, *Science*, 1955, v122, 501.

6. Gey, G. O., Coffman, W. D., and Kubicek, N. T., *Cancer Res.*, 1952, v12, 264.

7. Lutz, B. R., Fulton, G. P., Patt, D. I., Handler, A. H., and Stevens, D. F., *ibid.*, 1951, v11, 64.

8. Toolan, H. W., *ibid.*, 1954, v14, 660.

9. Furth, J., Gadsden, E. L., and Upton, A. C., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 253.

10. Handler, A. H., *Proc. Amer. Assoc. Cancer Res.*, 1955, v2, 22.

11. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

12. Handler, A. H., and Foley, G. E., to be published.

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Effects of Temperature and Work on Metabolism and Heat Loss in Man.*† (22225)

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Muscular exercise in a cold environment by either man(1) or animals(2,3) requires a greater oxygen consumption than the same activity in a warm environment. In man, the added energy requirement for work in the cold is not entirely the added hampering effect of clothing(4). Hart(2) concluded that the extent to which heat produced by activity is used to maintain body temperature depends upon the effect of activity on over-all physiological body insulation. The following experiments were devised to disclose changes of insulation in man exercising in warm and cool environments.

Methods. Skin temperature, heat loss and oxygen consumption were measured in 4 male subjects clad in shorts and exercising in cooling and cool environments. The experiments were begun at an ambient temperature of 20°C. The temperature was then steadily decreased to 10°C over ½ hour, and maintained at 10°C during the remainder of the test period (30 to 60 minutes). The subject reported shivering on a 4-point scale. Exercise on a bicycle ergometer was performed at a rate of 150 kg m/min or 70 kg m/min. Exercise was initiated with the start of cooling in some experiments, and after shivering had begun as a result of cooling in others. Skin temperature was measured with copper-constantan thermocouples recorded by a Brown

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† The authors appreciate the assistance of William Scheyer and Walter Cottle in conducting these experiments.

potentiometer with a −0.5 to 2.5 mV range. Thermocouples were placed according to the recommendations of Hardy and Du Bois(5), and the thermocouples were paralleled to give an average reading. Heat loss was recorded with Hatfield-Turner discs(6), 4 in series on the thorax and 4 in series on the thigh and leg. Oxygen consumption was measured with a laminar flowmeter(7) with a proportionate sample analyzed in a Pauling oxygen meter, range 110 to 160 mm Hg.

Results. The time course of the parameters measured is given in Fig. 1 and 2, and summarized in Fig. 3. When exercise was initiated during cooling (Exercise During Cooling, Item I, Table I), the skin temperature dropped at a faster rate over the initial 30 minute period than it did during cooling at rest (Item 2, Table I), although the final average skin temperature was slightly higher. The heat debt, calculated on the basis that ⅓ of the body weight was shell, was less when the subject exercised during cooling. Exercise during cooling was accomplished with an oxygen consumption comparable to that required when exercise was performed at 20°C (Item 4, Table I). Rectal temperature re-

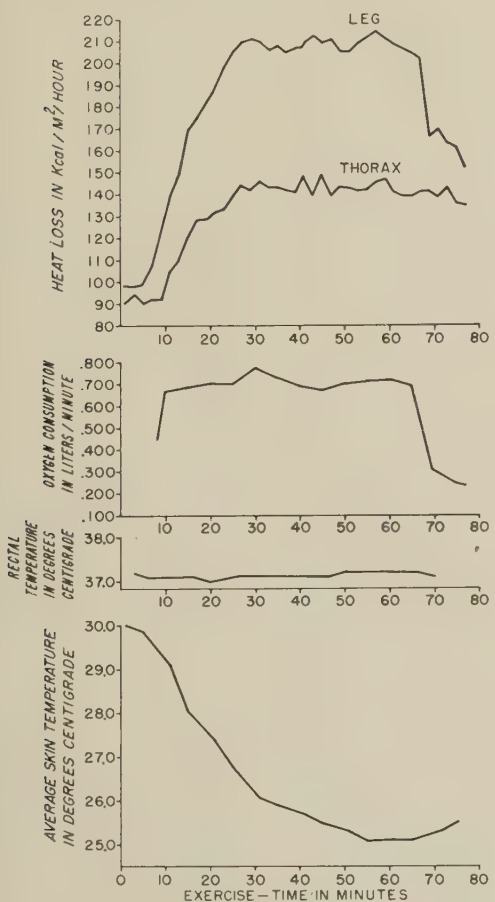

FIG. 1. Change in rectal temp., avg skin temp., oxygen consumption and heat loss with time. Cooling started at 5 min. Temperature changed from 20°C to 10°C over a ½ hr period and maintained at 10°C the remainder of the test period. Exercise started at 5 min. In the discussion this experiment is referred to as exercise during cooling. Avg of 4 subjects.

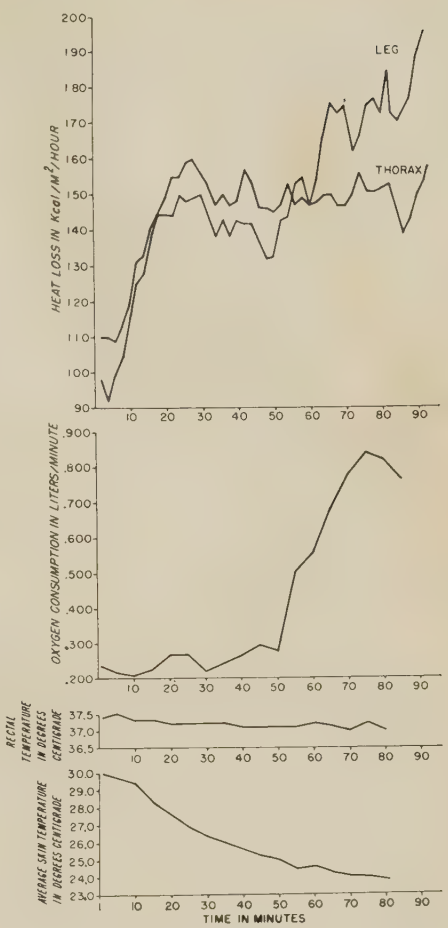

FIG. 2. Change in rectal temp., avg skin temp., oxygen consumption and heat loss with time. Cooling started at 5 min. Temperature changed from 20°C to 10°C over a ½ hr period and was maintained at 10°C for remainder of test period. Exercise was initiated at 50 min. In the discussion this experiment is referred to as rest during cooling and exercise after cooling. Avg of 4 subjects.

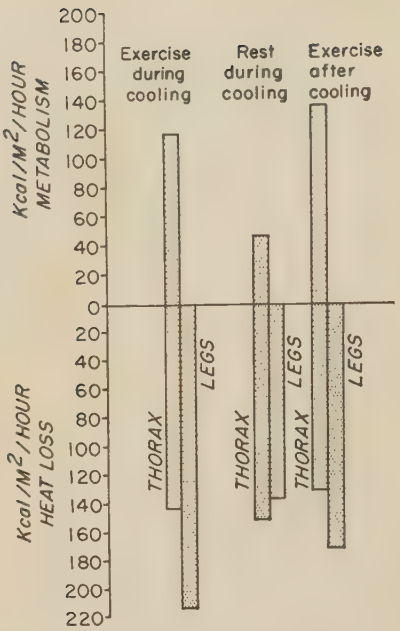

FIG. 3. Avg values for heat production plotted above horizontal line, and heat loss from the thorax and legs plotted below horizontal line. Heat loss from legs with exercise during cooling is greater than with exercise after cooling, and energy requirement when exercise is initiated after cooling is greater than when exercise is accomplished during cooling.

mained unchanged during the experiment when there was exercise during cooling, but fell slightly during cooling at rest.

Heat input increased when exercise was initiated after cooling, and the heat loss from the legs was not as great in this circumstance as when there was exercise during cooling. The increase in heat loss from the legs with exercise reflects the change in convective losses as well as the increased leg skin temperature. If the air insulation, Ia, is calculated for thorax and thigh plus leg according to the equation $Ia = \frac{Ts - Ta}{H}$ (8) this value for the thorax in the 2 experimental conditions is 0.12°C/cal/hr/m². The value of Ia for the legs is 0.105 during shivering and 0.067 to 0.081 during exercise.

Fall in rectal and skin temperature was arrested during exercise, but skin temperature did not rise during 20 min. of exercise. Increased heat loss via respiration is estimated to be of the order of 20% greater when exercise was initiated after shivering (29 kg cal/hr vs. 24 kg cal/hr).

Even after 90 min of shivering (Item 3, Table I), a steady state was not reached, and the heat input was approximately doubled. The skin and rectal temperatures were still slowly declining. The heat output did not balance with the heat input. At this time, the metabolism was 48 kg cal/hr/m² above the resting level at 20°C. Exercise, which at 20°C required a heat input of 45 kg cal/hr/m², required an additional 76 kg cal/hr/m² and then did not stop shivering in 30 min.

The drop in skin temperature may be used to calculate the heat debt on the basis that ⅓ of the body weight was cooling at this rate with a specific heat of 0.83. If the skin temperature decline is divided into 2 phases, the initial rapid decline and the subsequent slower fall, the heat debts may be compared. During cooling at rest heat debt was initially incurred at the rate of 75 kg cal/hr/m², and during cooling with exercise, at 93 kg cal/hr/m². In the second phase, skin temperature dropped an average of 3.9°C an hour at rest, and only 2.1°C an hour during exercise. Calculated on the same basis, this represents a rate of heat loss of 34 kg cal/hr/m² at rest and 18 kg cal/hr/m² during exercise. The area represented by the Hatfield-Turner discs on the thorax was assumed to be 0.35 body surface, and the area represented by the discs on the leg was assumed to be 0.32 body surface; Table II was then constructed. Considering the perils of the assumptions, the agreement of values obtained during exercise is particularly good.

Discussion. It appears from these data that the energy cost of exercise is greater after than during cooling. The reason is not demonstrated. Shivering usually continues during the exercise. The over-all direct heat loss, as indicated by the Hatfield-Turner discs, was less when exercise was performed after cooling. It would appear that the control of peripheral circulation exerted by exercise is modified by prior cooling. There may be an increased work related to the changes in temperature and motility that occur in muscles

TABLE I. Metabolic Changes with Exercise at Low Temperature.

	Rate of cooling, °C/min.	Time to shiver, min.	Skin temp., °C Initial	Skin temp., °C Final	Heat debt*	Heat input,† rest, 20°C	Heat input, shivering	Heat input, exercise	Heat loss, shivering	Heat loss, exercise	Heat loss, shivering	Heat loss, exercise
					kg cal/hr/m²							
Item 1												
Exercise during cooling	.16	—	30.2	25.5	49			122		145		223
	.13	—	29.8	24.5	57			112		149		183
	.14	—	29.8	24.7	53			110		146		224
	.17	—	30.5	25.5	64			120		132		206
Avg	.15	—	30.1	25.0	56	36		117		143		214
Item 2												
Cooling at rest; exercise after	.16	13	30.8	24.7	63		48	140	151	147	136	174
	.11	49	30.0	23.9	74		37	133	126	130	122	147
	.12	34	29.9	24.2	66		60	132	143	149	142	185
	.10	66	29.7	23.3	83	36	37	135	181	160	149	179
Avg	.12	48	30.1	24.3	72		46	135	151	147	137	171
Item 3												
Same as 1 & 2 (see ‡)	.18	33–48	31.5	25.0	67			83‡		182		223
	.15	25	30.7	25.0	60	35	79	111‡	170	168	134	189
Item 4												
Exercise at 20°C	0							107				
	0							80‡				

* Calculated on basis of 1/3 body cooling at skin temperature and 2/3 at rect. temperature.
† " " " " caloric value of liter of O_2 = 4.825.
‡ Exercise at 70 kg M/min., others at 150 kg M/min.

TABLE II. Heat Balance Calculations.

Item 1	Heat loss, kg cal/hr		Heat input, kg cal/hr					
	Thorax	94	Metabolism	221				
	Legs	128	Skin temp.	42				
	Remainder	41	Rectal	0				
		263		263				
Item 2	Heat loss (shivering), kg cal/hr		Heat input (shivering), kg cal/hr		Heat loss (exercise), kg cal/hr		Heat input (exercise), kg cal/hr	
	Thorax	100	Metabolism	87	Thorax	97	Metabolism	255
	Legs	82	Skin temp.	79	Legs	105	Skin temp.	0
	Remainder	0	Rectal	16	Remainder	49	Rectal	−4
		182		182		251		251

and joints(9,10). In these experiments, the steady state was not reached during the first 2 or 3 hours of cooling. Metabolism was eventually doubled at 10°C. In these experiments skin temperature dropped at a rate comparable to that reported by Horvath *et al.* (11), whose subjects were well-clothed individuals exposed to −40°C with a heat input of 74 kg cal/hr/m^2 at 2 hours' and 85 kg cal/hr/m^2 at 3 hours' exposure. The increase in heat input with exercise is of the same order of magnitude as that reported by Horvath for clothed men.

Summary. Exercise after cooling is accomplished at a greater energy cost than exercise during cooling. Over-all direct heat loss is less during exercise after cooling.

1. Horvath, S. M., Freedman, A., and Golden, H., *Am. J. Physiol.,* 1947, v50, 99.

2. Hart, J. S., *Canad. J. Zool.,* 1952, v30, 90.

3. Hart, J. S., and Heroux, O., *Canad. J. Biochem. & Physiol.,* 1955, v33, 428.

4. Horvath, S. M., and Golden, H., *J. Clin. Invest.,* 1947, v26, 311.

5. Hardy, J. D., and Du Bois, E. F., *J. Nutrition,* 1938, v15, 477.

6. Hatfield, H. S., *J. Sci. Instruments,* 1950, v27, 1.

7. Carlson, L. D., *Methods in Med. Res.,* 1954, v6, 60.

8. Gagge, A. P., Burton, A. C., and Basett, H. C., *Science,* 1941, v94, 428.

9. Hunter, J., and Whillans, M. G., *Canad. J. Med. Sci.,* 1951, v29, 255.

10. Hunter, J., Kerr, E. H., and Whillans, M. G., *ibid.,* 1952, v30, 367.

11. Horvath, S. M., Golden, H., and Wager, J., *J. Clin. Invest.,* 1946, v25, 709.

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Phosphatase Activity of Hematopoietic Tissues of Nitrogen Mustard-Treated Animals.* (22226)

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Previous studies in this laboratory(1) have shown that whole body x-irradiation markedly inhibits citric acid synthesis *in vivo* by the spleens and thymus glands of rats and mice. Treatment of animals with nitrogen mustards caused a similar inhibitory effect on citric acid formation by hematopoietic tissues (2). More recently we found(3) that sub-lethal and lethal doses of x-irradiation produce marked increases in the adenosine triphosphatase and 5-nucleotidase activity of the spleens and thymus glands. In view of the known similarities between x-irradiation and the nitrogen mustards in some of their actions on mammalian tissues we were interested in ascertaining whether these chemical agents produced changes in phosphatase activity similar to those which

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result from x-irradiation. To test this possibility various doses of the hydrochlorides of methyl *bis*(2-chlorethyl)amine (HN2) and ethyl *bis*(2-chlorethyl)amine (HN1) were administered intraperitoneally to rats and the adenosine triphosphatase and 5-nucleotidase activity of the spleens and thymus glands was measured at various intervals. Some measurements of the adenosine triphosphatase activity of the tissues of mice treated with HN2 were also made. The results of these experiments demonstrated that the nitrogen mustards resemble x-irradiation(3) in their effects on the phosphatase activity of hematopoietic tissues.

Materials and methods. Young male Sprague-Dawley rats (175-225 g) and male Carworth Farms mice (20-30 g) were used for this study. The animals were maintained in an air-conditioned laboratory at 65-75°F and were fed Rockland Rat Diet. Freshly prepared aqueous solutions of HN1 and HN2 were administerd intraperitoneally. For the enzyme assays the animals were sacrificed by decapitation at intervals after treatment with the nitrogen mustards and cold, aqueous homogenates of spleen and thymus glands were prepared. The adenosine triphosphatase assays were performed by the method of DuBois and Potter(4) using 1% homogenates of mouse spleen, mouse thymus glands and rat thymus glands and 0.5% homogenates of rat spleen. Duplicate assays using 2 levels of tissue were made. For the measurements on rat spleen 0.5 mg and 1 mg of tissue were used and for the assays on the thymus glands of both species and mouse spleen 1 mg and 2 mg of tissue were employed. The adenosine triphosphatase activity was expressed as units, *i.e.*, the micrograms of inorganic phosphorus liberated from adenosine triphosphate/mg of tissue during a 15-minute incubation period. The 5-nucleotidase assays were performed using a method developed in this laboratory(5). For measurement of the 5-nucleotidase activity of rat spleen and thymus glands 2% homogenates were prepared and quantities of homogenate containing 2 mg and 4 mg of tissue were used. Inorganic phosphorus measurements were made by the method of Fiske and SubbaRow(6).

Results. In a previous study in which HN1 and HN2 were administered to rats under conditions comparable with those used for the present experiments the LD_{50} of the hydrochloride of HN1 was 0.75 mg/kg and LD_{50} for HN2 hydrochloride was 1.5 mg/kg when the compounds were given intraperitoneally (2). To measure the influence of HN2 on the adenosine triphosphatase activity of the spleens and thymus glands of rats sublethal doses of 0.5 mg/kg and 0.75 mg/kg were given to 2 series of animals. At daily intervals for 5 days 4 animals at each dosage level were sacrificed for adenosine triphosphatase measurements on the spleens and thymus glands. The results of these measurements are shown in Fig. 1 in which each value on the curves is the average for assays on the tissues of 4 animals.

Administration of 0.75 mg/kg of HN2 hydrochloride resulted in an increase in the adenosine triphosphatase activity of the spleen to 186% of normal in 2 days. The spleens of rats which received 0.5 mg/kg of HN2 hydrochloride exhibited a maximum increase to 161% of normal in 3 days. Gradual reversal of the alteration in enzyme activity followed the initial increase and the rate of reversal was more rapid with the lower dose of HN2. The thymus glands exhibited a much smaller increase in enzyme activity during the observation period after these doses of the nitrogen mustard. However, in other experiments in which higher doses of HN2 were administered considerable increases in the adenosine triphosphatase activity of the thymus glands were noted. Thus, after 2 mg/kg of HN2 hydrochloride the adenosine triphosphatase activity of the thymus glands increased to 179% of normal in 3 days.

TABLE I. Comparison of the Increase in Adenosine Triphosphatase Activity of Spleens of Rats 72 Hours after Various Doses of HN1 and HN2.

Nitrogen mustard (mg/kg)	Adenosine triphosphatase activity (μg P/mg tissue/15 min.) HN1	HN2
.5	36.4 (34.3-38.0)	27.5 (26.4-28.9)
.75	44.6 (40.1-46.5)	30.3 (28.2-32.5)
1.0	47.4 (43.5-49.6)	35.6 (34.4-37.6)

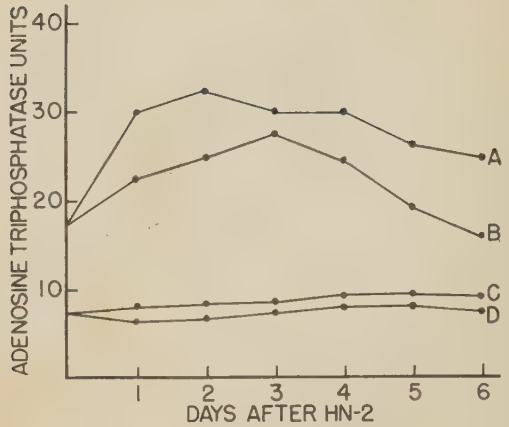

FIG. 1. Effect of methyl *bis*(2-chlorethyl)amine (HN2) hydrochloride on adenosine triphosphatase activity of spleens and thymus glands of rats. A, spleen 0.75 mg/kg; B, spleen 0.5 mg/kg; C, thymus glands 0.75 mg/kg; D, thymus glands 0.5 mg/kg.

To ascertain whether a relationship exists between the toxicity of nitrogen mustards and their ability to increase the adenosine triphosphatase activity of the spleens of rats a comparison of the effects of equivalent doses of HN1 and HN2 was made. For these measurements rats were given 0.5, 0.75 and 1 mg/kg of the hydrochlorides of HN1 and HN2 intraperitoneally. Groups of 4 animals at each dosage level were sacrificed at 72 hours for adenosine triphosphatase measurements on the spleens. The results of these measurements are shown in Table I in which the average and the range of values for each group are presented. The amount of increase in adenosine triphosphatase activity produced by each of the nitrogen mustards was dependent upon the dose and HN1 was more effective than HN2 at comparable dosage levels in agreement with the higher toxicity of HN1 to rats.

The effects of HN2 on the adenosine triphosphatase activity of mouse tissues was measured after administration of single doses of 2, 5 and 10 mg/kg of the hydrochloride intraperitoneally. The results of adenosine triphosphatase measurements conducted at daily intervals on the spleens and thymus glands are summarized in Fig. 2 in which each value on the curves is an average for at least 4 animals. At 3 days after administration of 5 and 10 mg/kg of HN2 hydrochloride the adenosine triphosphatase activity of the spleens of mice increased to 165% and 186% of normal respectively. Similar increases were noted in the thymus glands. The respective mean values and ranges for the spleens of mice receiving the 2 doses of HN2 were 18.2 (15.7-19.9) and 20.5 (17.4-23.6) adenosine triphosphatase units. Statistical analysis of the difference between these values yielded a t value of 2 indicating that the difference is of doubtful significance. Thus a dose of 5 mg/kg produced the maximum increase in the adenosine triphosphatase activity which can be induced by HN2 in the spleens and thymus glands of mice. A similar effect was observed in rats in that doses of 2.5 and 5 mg/kg of HN2 hydrochloride caused essentially the same amount of increase in the adenosine triphosphatase activity of the hematopoietic tissues. Dose-dependent increases in phosphatase activity are, therefore, observed mainly in the sublethal dosage range after administration of the nitrogen mustards or exposure to x-ray(3). Another series of mice received 2 mg/kg of HN2 hydrochloride which is sublethal for this species. The adenosine triphosphatase activity of the spleen increased to an average value of 156%

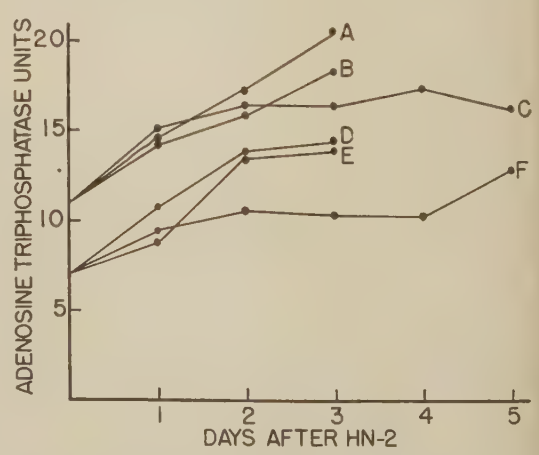

FIG. 2. Influence of methyl *bis*(2-chlorethyl)amine (HN2) hydrochloride on adenosine triphosphatase activity of spleens and thymus glands of mice. A, spleen 10 mg/kg; B, spleen 5 mg/kg; C, spleen 2 mg/kg; D, thymus glands 10 mg/kg; E, thymus glands 5 mg/kg; F, thymus glands 2 mg/kg.

of normal in 4 days after which time a gradual reversal toward normal levels occurred.

Measurements of the influence of HN2 on the 5-nucleotidase activity of the spleens and thymus glands of rats were also conducted. For this experiment a series of rats was given 0.5 mg/kg of HN2 hydrochloride intraperitoneally and groups each containing 4 animals were sacrificed at daily intervals for 5 days. The 5-nucleotidase activity of the spleens increased to 153% of normal in 4 days and an increase to 167% of normal was noted in the thymus glands at 3 days after administration of the nitrogen mustard. Gradual return of the enzyme activity to normal levels followed the initial increase in activity. The intraperitoneal administration of 0.75 mg/kg of HN2 hydrochloride caused an increase in the 5-nucleotidase activity of the spleen to 173% of normal and an increase in the enzyme activity of the thymus glands to 149% of normal.

Discussion. Results of this investigation demonstrated that sublethal doses of the nitrogen mustards produce increases in the adenosine triphosphatase and 5-nucleotidase activity of the spleens and thymus glands. The amount and duration of the increase in enzyme activity was dependent upon the dose of the nitrogen mustards. Higher doses of HN2 were necessary to produce increases in the adenosine triphosphatase activity of the hematopoietic tissues of mice than rats in agreement with the higher toxicity of this nitrogen mustard to rats. At equivalent dosage levels HN1 produced a greater increase in adenosine triphosphatase activity than HN2 thus indicating a relationship between the toxicity of various nitrogen mustards and their actions on phosphatase activity *in vivo*. After sublethal doses of the nitrogen mustards the increase in enzyme activity was reversible. The effects of the nitrogen mustards on the phosphatase activity of the spleens and thymus glands resembled qualitatively the effects of x-ray on the phosphatases of these tissues. This relationship was evident with respect to time of onset, magnitude and duration of the increase in enzyme activity. However, from a quantitative standpoint x-ray was more effective in terms of the LD_{50} values than the nitrogen mustards in increasing the phosphatase activity of hematopoietic tissues.

Previous studies in this laboratory have demonstrated(1,2) a qualitative similarity between the ability of the nitrogen mustards and x-ray to inhibit citrate synthesis in hematopoietic tissues of rats. On the basis of the available evidence it is not possible to state whether the defect in citric acid synthesis is related to the alteration in phosphatase activity. Both of these biochemical changes occur at essentially the same time and persist for approximately equal periods after treatment of animals with x-ray or a nitrogen mustard. The exact mechanism involved in the production of increased phosphatase activity in the hematopoietic tissues produced by these toxicants is unknown. On the basis of histochemical findings Ackerman *et al.*(7) suggested that the radiation-induced increase in phosphatase activity of the spleen is due to a relative increase in the red pulp and clasmatocytes as well as an actual increase in the phosphatase of the clasmatocytes. The similarity between the nitrogen mustards and x-ray which was demonstrated in the present investigation indicates that either of these agents may be employed in future studies on the exact mechanism responsible for the increased phosphatase activity of hematopoietic tissues.

Summary. The effects of nitrogen mustards *in vivo* on the phosphatase activity of the spleens and thymus glands of rats and mice were studied. The results of this study indicated that sublethal doses of HN2 produce dose-dependent increases in the adenosine triphosphatase activity and the 5-nucleotidase activity of the spleens and thymus glands with the maximum increase being observed at 3 or 4 days after intraperitoneal administration of the compound. Reversal of the increase in enzyme activity was noted after sublethal doses of HN2. At equivalent dosage levels HN1 was more toxic and more effective than HN2 in increasing the adenosine triphosphatase activity of the spleens and thymus glands of rats.

1. DuBois, K. P., Cochran, K. W., and Doull, J.,

PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 422.

2. DuBois, K. P., Deroin, J., and Cochran, K. W., *ibid.*, 1952, v81, 230.

3. DuBois, K. P., and Petersen, D. F., *Am. J. Physiol.*, 1954, v176, 282.

4. DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, v150, 185.

5. Cochran, K. W., Zerwic, M. M., and DuBois, K. P., *J. Pharm. Exp. Therap.*, 1951, v102, 165.

6. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1926, v66, 375.

7. Ackerman, G. A., Bellios, N. C., Knouff, R. A., and Frajola, W. J., *Blood*, 1954, v9, 795.

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Biotin Derivatives in Human Urine. (22227)

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The nature of the biotin derivatives that occur in urine has been the subject of a number of studies since Snell *et al.* (1) first showed that urine is a relatively rich source of the vitamin. Oppel(2) concluded that there exist in urine two forms of biotin, one avidin combinable and one avidin non-combinable. Burk and Winzler(3) postulated the existence in natural material (including urine) of a number of biotin derivatives (miotin, tiotin, and rhiotin) distinguishable by differences in microbiological activity, chemical stability, or avidin combinability. Chu and Williams(4), on the other hand, could not detect the presence in any natural material other than urine of avidin non-combinable forms of biotin. They even seriously questioned the real validity of their own data concerning the existence in urine of small amounts of such a derivative(s).

The above described studies were carried out primarily with *Saccharomyces cerevisiae* as the assay organism. Since yeast growth is notoriously influenced by a variety of stimulatory and inhibitory materials of unknown nature, the specificity of the responses described above with respect to any real relationship to biotin may be questioned. Significant to this discussion also is the fact that none of the compounds whose existence was postulated in the references cited has ever been obtained in the pure or even highly concentrated state. On the contrary, a number of other biotin derivatives have been derived from natural material or products derived from natural material and have been adequately characterized. These derivatives are biocytin (ε-N-biotinyl-L-lysine)(5), biotin d-sulfoxide(6-8), and biotin l-sulfoxide(9-11). A number of other biotin derivatives including biotinamide(12,13), oxybiotin(14), and N-biotinyl derivatives(12,13) of glycine, β-alanine, L-aspartic acid, L-glutamic acid, L-leucine, and p-aminobenzoic acid have been synthesized in the laboratory and studied microbiologically. Differential microbiological assays with a high order of specificity in some instances are now available for studies with these compounds. In combination with the bioautographic technic, such differential microbiological asays can yield considerable information concerning the nature of the biotin derivatives in a given sample. One study(15) employed butanol-acetic acid-water as the developing system and *Neurospora crassa* as the indicating organism. The butanol-acetic acid-water system is remarkably free of complications from salts and other components of natural material. The organism *Neurospora crassa* is extremely versatile as far as response to biotin derivatives is concerned. The following compounds are essentially equally active: Biotin, oxybiotin, biotinamide, desthiobiotin, biotin d-sulfoxide, biotin l-sulfoxide, biocytin, biotinyl β-alanine, biotinyl glycine, biotinyl aspartic acid, and biotinyl leucine(10,12,13,16). In addition, R_F data obtained with *Neurospora crassa* are available for the mixed sulfoxides or the individual d- and l-sulfoxides in some

instances of biocytin, biotinyl β-alanine, biotinyl glycine, biotinyl aspartic acid and biotinyl leucine which are formed when these compounds are paper chromatographed in those solvents which lead to the similar formation of biotin d-sulfoxide and biotin l-sulfoxide from biotin(15,16). Pimelic acid and related dicarboxylic acids are inactive microbiologically. On the other hand, *Lactobacillus arabinosus* is highly specific with respect to its requirement for biotin. Oxybiotin, N-biotinyl glycine, and biotin d-sulfoxide are the only compounds among the biotin derivatives presently available with more than 5% of the activity of biotin in promoting growth of this organism. The difference btween the "biotin" content of a particular sample determined with *Neurospora crassa* and that obtained with *Lactobacillus arabinosus* would appear then to have some significance as a measure of derivatives of biotin. The cyclic urea ring of biotin is essential for avidin combinability. Biotin derivatives containing such a structure combine with avidin with widely varying but characteristic affinities that are always less than that found with biotin itself(17,18). Cognizance of the above considerations has been made in the present studies on the biotin content of human urine.

Materials and methods. "Free" biotin was determined in the urine samples with *Lactobacillus arabinosus* (ATCC 8014) as described previously in detail(19,20). "Total" biotin was determined with *Neurospora crassa*(9,11). The bioautographic procedures also have been adequately described previously(9,11,15). In the present studies 0.2 ml of urine (0.02 ml applied 10 times) or 0.02 γ of biotin or biocytin as biotin (0.02 ml of a 1 γ/ml solution) or 2 mg of urea (0.02 ml of a 20 mg per ml solution applied 5 times) was chromatographed on Whatman No. 1 paper where the solvent phase was obtained from the equilibration of butanol(5), water(4), and acetic acid(1), and the aqueous phase was present in a separate receptacle. After removal of the solvent by air drying in the hood the paper chromatograms intended for bioautography were cut into 21 sections such that each section represented 0.05 of an R_F unit. These sections were individually eluted in Erlenmeyer flasks with 25 ml of basal *Neurospora crassa* medium. After removal of the paper sections following a 1-hour elution period the flasks were plugged, autoclaved, seeded with *Neurospora crassa,* and incubated at 30° for 3-4 days. The extent of growth of the mold was determined by collecting the mycelium on a wire loop, squeezing to incipient dryness, and then drying to constant weight at 80°C (usually 18 hours). When the mycelium weight was plotted as a function of the paper section number a smooth curve connecting the points permitted localization of the R_F values by interpolation to within 0.02 unit. Urea was located on paper chromatograms by spraying with 2% p-dimethylaminobenzaldehyde in 1 N HCl. The avidin combinability of the biotin derivatives in urine was determined with *Neurospora crassa.* A microbiological assay using the usual 8 flasks of biotin standard, 2 series of 4 flasks containing increasing levels of urine, and 2 series of 8 flasks each containing a constant amount of the 2 urine samples studied was prepared. After the usual addition of medium, autoclaving, and seeding, 8 increasing levels of a sterile egg white dilution as a source of avidin were added in duplicate to the series of flasks to which the constant levels of urine had been added previously. After growth of the organism the weight of mycelium in each flask was determined in the usual way.

TABLE I. Biotin Derivatives in 4 Urine Samples.

"Total" biotin by *Neurospora crassa* assay, γ/ml	"Free" biotin by *Lactobacillus arabinosus* assay, γ/ml	Unknown biotin derivative by difference, γ/ml
.018	.016	.002
.018	.017	.001
.014	.012	.002
.029	.024	.005

Results. As indicated by the microbiological assay data of Table I human urine contains in addition to free biotin, or material with microbiological activity for *Lactobacillus arabinosus,* small amounts of a biotin derivative measurable with *Neurospora crassa.*

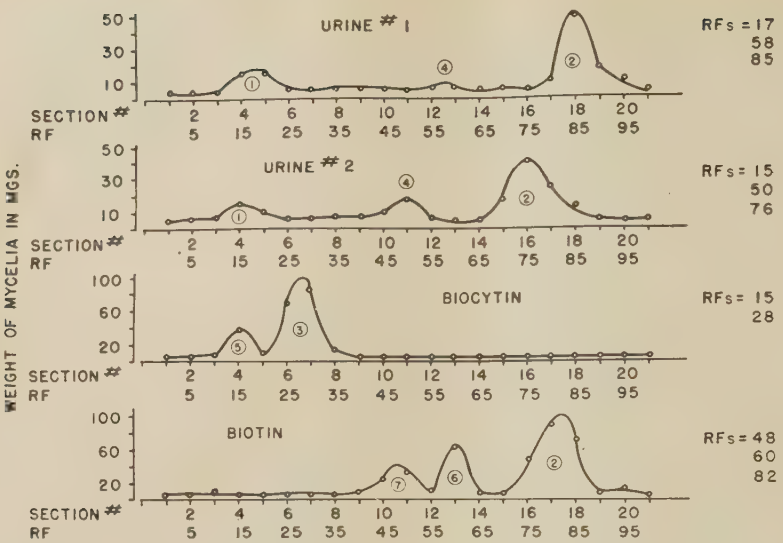

FIG. 1. Summary of bioautographic data. The various peaks are interpreted as follows: (1) unknown biotin derivative, (2) biotin, (3) biocytin, (4) biotin "sulfoxide," (5) biocytin "sulfoxide," (6) biotin d-sulfoxide, (7) biotin l-sulfoxide.

The paper chromatographic data of Fig. 1 confirm the predominant form of biotin in urine measurable with *Lactobacillus arabinosus* as free biotin (R_F ca 0.83). Indicated also by the chromatograms is the presence of biotin d-sulfoxide. This compound together with much smaller amounts of biotin l-sulfoxide (not present in detectable amounts in the present urine chromatograms) is encountered when biotin is paper chromatographed on Whatman No. 1 paper in the butanol-water-acetic acid system. Its presence in the chromatogram when biotin is also present does not necessarily mean that it was originally present in the urine samples studied. The paper chromatograms do indicate the existence in urine of small amounts of a biotin derivative with a low R_F value. The unknown material has an R_F value in the butanol-water-acetic acid system corresponding quite closely to that of the two sulfoxides of biocytin which are not resolved in this solvent or other solvent systems studied. The corresponding sulfoxide of biocytin to which the present biotin derivative conceivably may be related is unknown, and accordingly is referred to hereafter as "biocytin sulfoxide." R_F values for the available N-biotinyl derivatives of glycine, β-alanine, aspartic acid, and leucine as well as the corresponding sulfoxides are all considerably higher(16). Attempts to demonstrate correspondence of R_F values of the unknown component of urine and "biocytin sulfoxide" in a number of other solvent systems have not been conclusive since it became apparent that the R_F values observed in these additional solvents were influenced to some extent by the extraneous components of urine. A certain amount of concentration and purification probably is indicated before paper chromatographic data would be conclusive regarding the identity of the biotin derivative of urine with "biocytin sulfoxide." It seems unlikely that, if the unknown derivative indicated on the chromatograms is "biocytin sulfoxide," it arose from an *in vitro* oxidation of biocytin by the filter paper for the following reasons: (a) it is present in urine prior to any contact with filter paper as indicated by the differential microbiological assays, and (b) biocytin, from which "biocytin sulfoxide" might conceivably have arisen by a certain amount of *in vitro* oxidation analogous to that observed with biotin, was entirely absent as indicated by the chromatograms. Biocytin would not be expected to occur in urine be-

TABLE II. Avidin Combinability of Biotin Derivatives in Urine.

Biotin, γ	Urine, ml	Egg white (avidin), ml	Mycelium, mg
0			1.0
.0005			11.6
.0010			19.7
.0015			27.7
.0020			31.9
.0030			42.6
.0050			45.0
	.02 No. 1		13.2
	.04		22.2
	.06		30.0
	.10		42.1
	.02 No. 2		10.0
	.04		17.3
	.06		24.9
	.10		36.8
	.1 No. 1	.002	24.6
	.1	.004	4.0
	.1	.006	1.5
	.1	.01	0
	.1	.02	0
	.1	.04	0
	.1	.06	0
	.1	.10	0
	.1 No. 2	.002	15.4
	.1	.004	3.2
	.1	.006	.7
	.1	.01	0
	.1	.02	0
	.1	.04	0
	.1	.06	0
	.1	.10	0

cause of the rapid hydrolysis of biocytin by an enzyme of blood (and presumably other tissues)(21). If the unknown component of urine is not "biocytin sulfoxide" it is at least a derivative considerably more "hydrophilic" than biocytin. Urea in lieu of biotin was found to be inactive for *Neurospora crassa* and to have an R_F value in the butanol-acetic acid-water system high enough (0.46) to eliminate it from consideration as a non-specific stimulant. As indicated by the data of Table II all the biotin in urine determinable with *Neurospora crassa* is combinable with avidin. These results would indicate that the unknown derivative contains a cyclic urea ring.

The best interpretation of the data described above is that with a biotin auxotroph considerably more versatile with respect to utilization of biotin derivatives than microorganisms previously employed but at the same time considerably more specific with respect to the response being due to material with a structural or functional relationship to biotin, no evidence is available for the existence in human urine of any "unusual" biotin derivatives. Although an unknown form of biotin is present the compound on the basis of paper chromatographic data could very well be "biocytin sulfoxide." No evidence is available for the existence of "avidin uncombinable" forms of biotin.

The isolation and characterization of the derivative according to classical procedures would be a formidable undertaking. Since the factor is present in urine only to the extent of about 2 γ per liter (provided, of course, that its microbiological activity is equal to that of biotin), to obtain the 100 mg that might be required for characterization studies, it would be necessary to work up at least 50,000 liters of urine. These calculations assume 100% recovery of the factor. If, as is more usual in such isolations, the recovery were to run 1 to 10%, the processing of between 500,000 and 5,000,000 liters of urine would be required for successful completion of the problem. When calculated in another way the factor may be described as present to the extent of only about 0.04 γ per g of urinary dry matter. If urine is the only source of the factor a comparison of the occurrence of this factor with the occurrence of other "trace factors" reveals that this compound is probably one of the most sparsely distributed of any biochemical compound presently known.

Summary. Microbiological experiments involving direct assays as well as bioautographic procedures indicate that human urine contains relatively large amounts of free biotin and very small amounts of a "hydrophilic" biotin derivative that may be "biocytin sulfoxide." No evidence was found for the existence of other "unusual" biotin derivatives. All the biotion of urine determinable by an organism more versatile in its response to biotin derivatives than any studied previously is avidin combinable.

1. Snell, E. E., Eakin, R. E., and Williams, R. J.,

J. Am. Chem. Soc., 1940, v62, 175.

2. Oppel, T. W., *Am. J. Med. Sci.,* 1942, v204, 856.

3. Burk, D., and Winzler, R. J., *Science,* 1943, v97, 57.

4. Chu, E. J-H., and Williams, R. J., *J. Am. Chem. Soc.,* 1944, v66, 1678.

5. Wright, L. D., Cresson, E. L., Skeggs, H. R., Peck, R. L., Wolf, D. E., Wood, T. R., Valiant, J., and Folkers, K., *Science,* 1951, v114, 635.

6. Melville, D. B., Genghof, D. S., and Lee, J. M., *Fed. Proc.,* 1950, v9, 204.

7. Melville, D. B., *J. Biol. Chem.,* 1954, v208, 495.

8. Melville, D. B., Genghof, D. S., and Lee, J. M., *ibid.,* 1954, v208, 503.

9. Wright, L. D., and Cresson, E. L., *J. Am. Chem. Soc.,* 1954, v76, 4156.

10. Wright, L. D., Cresson, E. L., Valiant, J., Wolf, D. E., and Folkers, K., *ibid.,* 1954, v76, 4160.

11. ———, *ibid.,* 1954, v76, 4163.

12. Wolf, D. E., Valiant, J., and Folkers, K., *ibid.,* 1951, v73, 4142.

13. Wright, L. D., Skeggs, H. R., and Cresson, E. L., *ibid.,* 1951, v73, 4144.

14. Pilgrim, F. J., Axelrod, A. E., Winnick, T., and Hofmann, K., *Science,* 1945, v102, 35.

15. Wright, L. D., Cresson, E. L., and Driscoll, C. A., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 480.

16. ———, unpublished data.

17. Wright, L. D., and Skeggs, H. R., *Arch. Biochem.,* 1947, v12, 27.

18. Wright, L. D., Skeggs, H. R., and Cresson, E. L., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 150.

19. Wright, L. D., and Skeggs, H. R., *ibid.,* 1944, v56, 95.

20. Wright, L. D., *Biological Symposia,* 1947, v12, 290.

21. Wright, L. D., Driscoll, C. A., and Boger, W. P., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 335.

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Interaction of Gelatin-Stabilized Radiogold Colloid and Plasma Proteins. (22228)

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It has been demonstrated that the rate of disappearance of gelatin-stabilized radiogold colloid (Aurcoloid) from the circulation is inversely proportional to the amount of gelatin used to stabilize the Aurcoloid(1). Furthermore, the gelatin effect was enhanced by either injection of homologous rat plasma or alpha and beta globulin fractions of human plasma, whereas human serum albumin and gamma globulin had little effect. There was no evidence to suggest that the phagocytic capacity of the reticulo-endothelial system (RES) for the gold particles had been altered by gelatin. The present study was undertaken to investigate the interactions of gelatin with the plasma proteins as a possible explanation for this effect of gelatin.

Materials and methods. The Aurcoloid* was diluted with 50 volumes of distilled, demineralized water and stored in a refrigerator. The P-111 gelatin† solution, similar to that used by the manufacturer to stabilize the Aurcoloid, was handled under sterile conditions and maintained at 37°C in a water bath for at least 15 minutes before use. For the *in vitro* experiments, blood was obtained from adult male rats of the Wistar strain via the abdominal aorta using heparinized syringes. Human blood was obtained by venipuncture using heparinized syringes. After centrifugation, 3 ml volumes of plasma were placed in test tubes to which was added 0.05 ml of the diluted Aurocoloid and 2.5 mg of gelatin. Plasma samples were agitated for 2 minutes, incubated at 37°C for 30 minutes, then electrophoretically separated. In the *in vivo* experiments, adult male rats of Wistar strain weighing between 200-300 g were used. Animals were anaesthetized by intraperitoneal injection of nembutal, 5 mg/100 g of body weight. The external jugular veins were exposed and injections of the Aurcoloid

* Obtained from Abbott Laboratories, Oak Ridge, Tenn.

† Obtained from Charles B. Knox Gelatin Products, Camden, N. J.

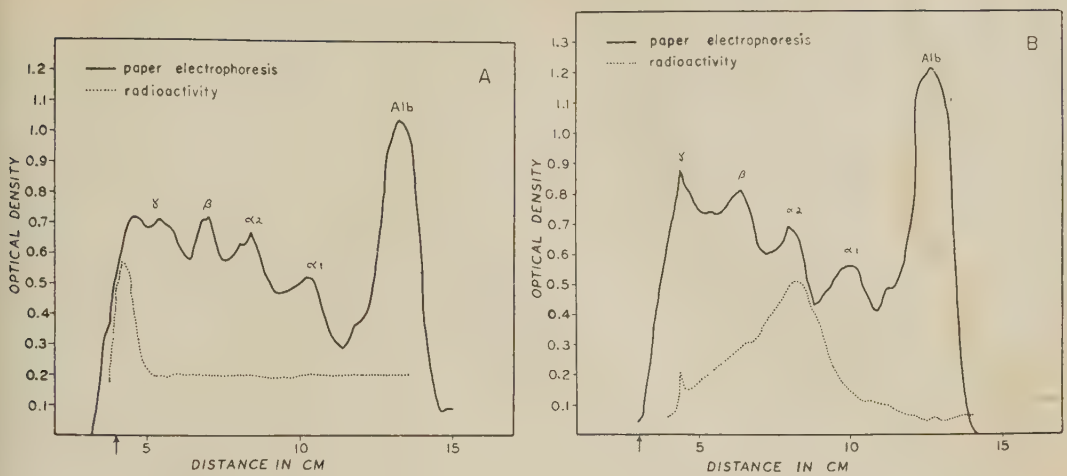

FIG. 1. Gelatin-plasma interaction *in vitro*. A. Human plasma plus radiogold. B. Human plasma plus gelatin and radiogold. Densitometric measurements of radioactivity made from radioautographs.

with and without added gelatin (2.5 mg/100 g of body weight) were given in one vein; blood samples (0.2 ml) were withdrawn from the opposite vein at various intervals using heparin coated syringes. Blood samples were centrifuged and the plasma was fractionated by paper electrophoresis. Paper electrophoresis was performed using a horizontal apparatus with the paper strips suspended by a central bridge. Four strips of Whatman 3 MM filter paper, 3.75 x 62 cm, were run simultaneously. Veronal buffer at pH 7.8 for rat plasma and pH 8.6 for human plasma and 0.05 ionic strength was used. After equilibration, 20 lambda of plasma was applied by streaking across a ruled line. A constant voltage of 100 volts was employed for 16 hours with a current of 4-5 milliamperes. After drying at 120°C for 30 minutes either radioautographs were made of the paper strips or the radioactivity on the strips was counted by a gas-flow chromatograph scanner driven by a synchronous mechanical feed from an Esterline-Augus recording apparatus. Thus the radioactivity patterns could be directly superimposed on the plasma protein patterns. The paper strips were then stained with bromphenol blue. Densitometry of the paper strips and radioautographs was performed with a Photovolt Model 501A instrument with optical density recorded at 2 mm intervals.

Results. When Aurcoloid alone was added to human plasma *in vitro,* the radioactive material did not migrate but remained at the origin. However, if gelatin and Aurcoloid were added to plasma the radiogold now had a mobility which corresponded with the alpha 2 globulin fraction (Fig. 1). Similar experiments using rat plasma gave the same results.

In the *in vivo* experiments the effect of gelatin was similar to that seen in the *in vitro* studies. Paper electrophoresis of plasma obtained from animals previously injected with gelatin and Aurcoloid demonstrated the similar location of the radioactive material and the alpha globulin fraction, whereas the radioactive gold did not migrate when Aurcoloid alone was injected (Fig. 2). The rate of disappearance of the Aurcoloid from the blood was markedly retarded in the gelatin injected animals (compare A and B, Fig. 2).

The relative mobilities of gelatin and the alpha globulin fractions of rat plasma were demonstrated by paper electrophoresis. Samples containing various combinations of Aurcoloid, gelatin and plasma were placed on filter paper and run simultaneously in the electrophoresis chamber. Aurcoloid alone, or in plasma, did not migrate. When Aurcoloid was added to gelatin the radiogold migrated with the gelatin whereas the combination of Aurcoloid, gelatin and plasma resulted in a

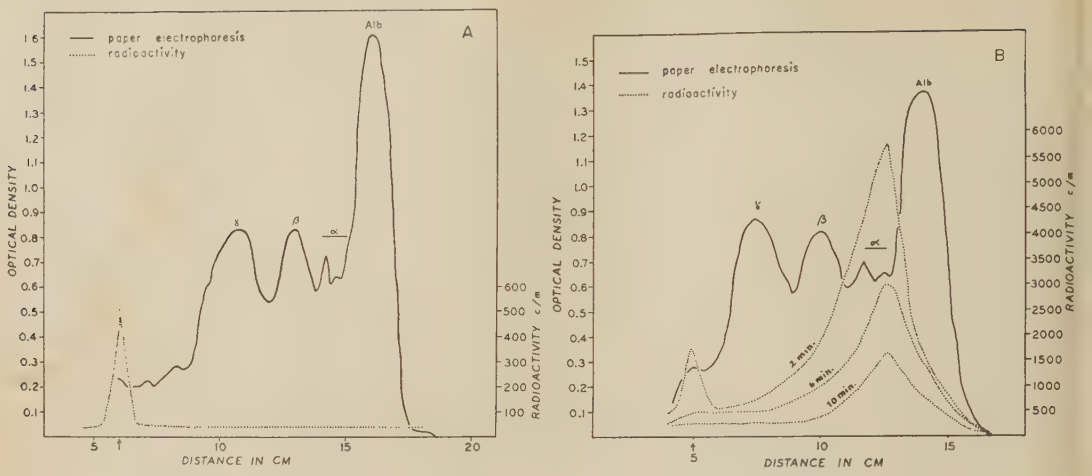

FIG. 2. Gelatin-plasma interaction *in vivo*. A. Blood sample removed 2 min. after radiogold injection. B. Blood samples removed at intervals after radiogold plus gelatin injection. Total radioactivity injected was similar in A and B.

still greater mobility of the radiogold which corresponded with the alpha globulin fraction (Fig. 3). These experiments suggested interaction between gelatin and the alpha globulin fraction and the possibility that radiogold particles are merely incorporated in the gelatin mass. The formation of a radiogold-gelatin-alpha globulin complex was associated with retention of radiogold in the circulation for longer intervals than was the case when interaction could not be demonstrated.

Discussion. Binding of radiogold colloid to the alpha and beta globulin fractions of human plasma, as demonstrated by Simon (2), may be explained by the fact that commercial radiogold preparations are stabilized by gelatin. The evidence presented in this investigation suggests that gelatin rather than

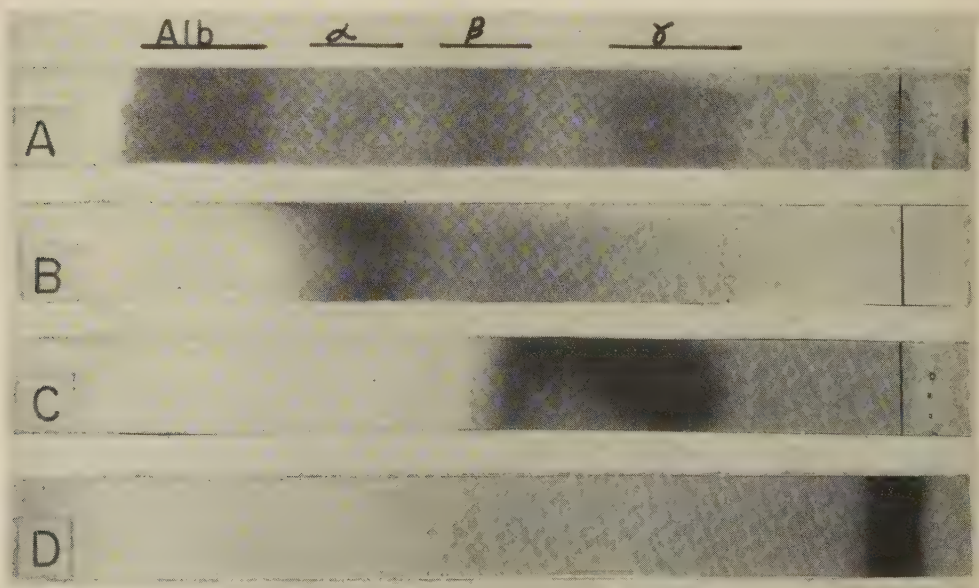

FIG. 3. Comparative electrophoretic mobilities of radiogold colloid. A. Electrophoretic pattern of rat plasma. B. Autograph: radiogold plus gelatin in plasma. C. Autograph: radiogold in gelatin. D. Autograph: radiogold in plasma.

metallic gold was bound by the alpha globulins. Although radiogold salts have been shown(3) to interact with the albumin fraction of plasma, Aurcoloid is relatively free of ionic gold.

The effect of gelatin in prolonging the retention of Aurcoloid in the circulation was apparently not mediated at the cellular level since colloidal gold was rapidly ingested by the RES regardless of whether the gold particles were coated with gelatin or plasma proteins(4). As Aurcoloid was eventually removed from the circulation by the RES, either the gold-gelatin-alpha globulin interaction was reversible or the total complex was phagocytised. Dissociation of the complex was not demonstrated in plasma electrophoretic patterns of animals injected with gelatin and Aurcoloid.

Summary. The relative mobilities of Aurcoloid and gelatin have been demonstrated *in vivo* and *in vitro* by paper electrophoretic methods. Addition of Aurcoloid and gelatin to plasma resulted in a radiogold-gelatin complex which migrated at the same rate as alpha globulins. Aurcoloid in plasma did not migrate. The formation of an Aurcoloid-gelatin complex was associated with a decreased rate of disappearance of radiogold from the circulation.

1. Murray, I. M., and Katz, M., *J. Lab. and Clin. Med.*, 1955, v46, 263.

2. Simon, N., *Science*, 1954, v119, 95.

3. Ekholm, R., *Acta Anat.*, 1951, v11, supp. 15.

4. von Jancso, N., *Z. f.d.ges., Exp. Med.*, 1929, v64, 256.

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Action of Parathyroid Extracts on Stable Bone Mineral Using Radiocalcium as Tracer.* (22229)

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When radiocalcium is injected into rats it becomes deposited in the skeletons of the animals in 2 forms which differ greatly in relative ease of mobilization and excretion. These forms of incorporation of radiocalcium and the parts of the skeletal bone mineral associated with each are referred to as the stable bone mineral and labile bone mineral(1,2). Fifty-two days after injection, radiocalcium is distributed almost exclusively in the stable fraction.

The present work describes the action of subcutaneously injected parathyroid extracts on radiocalcium (Ca^{45}) incorporated in the stable bone mineral.

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Procedure. Twenty-four adult-male rats of comparable age and weight (380-420 g) were given intraperitoneal injections of high specific activity radiocalcium. This was administered in physiological saline solution in 3 doses of 2 cc each, allowing 2 hour intervals between injections. The total injected dose amounted to approximately 9.9 x 10^5 counts per minute according to the methods of sample preparation and counting conditions employed. After 72 days the left foreleg of each rat was amputated at the shoulder. Within this time the radiocalcium had presumably been distributed preponderantly in the stable fraction of the skeleton(1,2). The humeri of the amputated legs were divested of adhering flesh and stored in a freezer to be compared with opposite pairs from the same animal obtained later at the time of sacrifice. One day after removal of the legs the rats were given a subcutaneous injection of 100 USP units of

Parathormone.‡ One group of rats received only a single injection and were sacrificed in pairs at intervals of 2, 4, 8, 14, and 24 hours after injection. The remaining rats received daily injections and were sacrificed in pairs at the end of 2, 3, 4, 5, 7, 9, and 13 days. The humeri of the right legs were obtained and then the ends of all humeri, including those from the amputated legs, were separated from the shafts by means of a rotating dental Carborundum disk, using recognizable anatomical landmarks. Epiphyseal and diaphyseal regions were then analyzed for Ca^{45} in order to detect any intra-skeletal shift of radiocalcium which might have occurred subsequent to the injection of Parathormone. Total calcium was also determined. Urine was collected daily from 6 of the rats housed in metabolism cages, and from 2 control rats. The control animals had undergone amputation but received isotonic saline injections instead of Parathormone. Radiocalcium was determined in the urine and humeri, using the method of sample preparation described by Armstrong and Schubert(3).

Results. The results of radioactivity measurements of the individual bone samples are recorded in Table I. Analysis for total calcium in these samples showed no differences either on a dry fat-extracted or ash basis. It is therefore convenient to express specific activity in terms of ash weight. It was found that no large replacement of radiocalcium in the stable bone mineral by non-radioactive calcium occurred, since the specific activities of experimental samples and the opposite leg control samples were practically identical in each case. The epiphyseal and diaphyseal regions of a given pair of bones were not significantly different with respect to specific activity either before or after Parathormone treatment, demonstrating that no major intrahumeral shift of radiocalcium occurred.

There was, however, a greatly increased movement of radiocalcium out of the skeleton as a result of stable bone mineral resorption associated with the large dosages of Parathormone. Fig. 1 shows the effect of Parathormone treatment on the daily renal excretion of radiocalcium released from stable bone mineral. In order to improve counting conditions, the daily urine volumes of 3 rats were pooled, by housing the 3 together in the same metabolism cage. Group A in Fig. 1 refers to a group of 3 rats receiving daily Parathormone injections, the results being expressed as percent of the injected dose excreted per rat per day. Group B refers to a parallel, entirely similar group of 3 rats receiving Parathormone daily, the urine volumes from which were also pooled for counting. The control value is based on the urinary analysis of 2 rats, with amputated legs, receiving subcutaneous saline injections instead of Parathormone. Prior to treatment of the experimental rats with Parathormone and in the control rats, the urinary excretion

TABLE I. Comparison of Ca^{45} in Epiphyseal and Diaphyseal Regions of Rat Humeri before and after Parathormone Injections, 23 Rats.

	Specific activity (% of injected dose/g of bone ash)			
	Epiphysis		Diaphysis	
Treatment	Control leg	Exp. leg	Control leg	Exp. leg
PTH,* single inj.				
2 hr	.66	.65	.71	.73
	.48	.45	.58	.59
4	.69	.66	.69	.70
	.57	.58	.62	.66
8	.61	.69	.69	.71
	.65	.64	.70	.69
14	.66	.64	.56	.62
	.62	.63	.69	.69
24	.56	.61	.65	.65
	.57	.53	.60	.59
PTH,* daily inj.				
2 days	.59	.56	.59	.60
	.63	.60	.65	.51
3	.61	.59	.65	.63
	.48	.48	.52	.51
4	.60	.57	.60	.43
	.67	.64	.66	.64
5	.57	.56	.61	.60
	.57	.59	.59	.60
7	.55	.54	.59	.55
	.55	.58	.57	.59
9	.65	.64	.64	.67
	.54	.59	.58	.59
13	.67	.62	.70	.71

* PTH = 100 USP units of Lilly Parathormone, inj. subcut.

‡ A gift of Eli Lilly Co., Indianapolis, Ind.

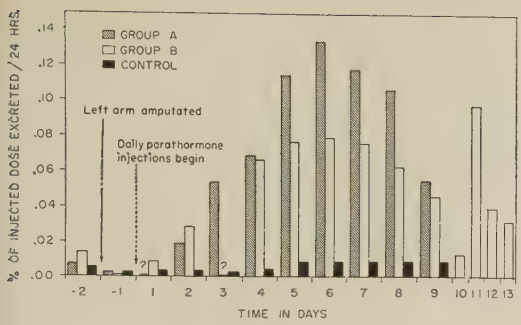

FIG. 1.

of Ca^{45} was between .009% and .013% of the injected dose per day. After a single Parathormone injection, radiocalcium in the urine was increased only slightly, but continued to increase during the first week of injections to a level of 0.08-0.14% (A). With repeated injections after the 6th day, however, the percentage of the injected dose in the urine began to decrease§ and after 13 days was down to .04%. In general this decline reflects the loss of Parathormone potency usually observed after repeated injections of bovine parathyroid extracts in normal rats(4).

Discussion. No decreases in the total calcium content or specific activity of radiocalcium in the experimental legs as compared to their previously amputated counterparts were observed. This might merely indicate that Parathormone injections mediate a complete withdrawal of the materials comprising bone, not acting upon calcium independently. It is possible, however, that the amount of material mobilized from the humerus, compared to the large amount present, is so small as to be undetectable by direct analysis of the bone. It is also possible, though unlikely, that radiocalcium appearing in the urine is derived to a lesser degree from the humerus than from other bones of the skeleton.

Unfortunately separation of the epiphyseal from the diaphyseal regions of the humeri, for the purpose of detecting intra-osteal shifts of Ca^{45}, resulted in variable losses of material. An accurate comparison between bone weights of amputated legs and experimental legs was thus prevented, but it would seem likely that the small amount of mineral mobilized from the vast skeletal reserves would not be detectable even to this method. The urinary excretion of radiocalcium appears to be the most sensitive indicator of a Parathormone effect on Ca^{45} deposited in the stable bone mineral.

The increased quantity of radiocalcium appearing in the urine shortly after initiation of daily injections of Parathormone indicates that these extracts influence the stable bone mineral to yield calcium to the body fluids. During 13 days of Parathormone treatment in rats, at least 60 times as much Ca^{45} was lost by renal excretion as in control animals.

The present experiments have an interesting correlation to a theory of parathyroid action recently formulated by McLean, who describes a dual mechanism for controlling the level of calcium in the plasma(5). One part, independent of the parathyroids, acts by simple chemical equilibrium with the labile fraction of the bone mineral to maintain a constant but low plasma calcium level of approximately 3.5 meq/l. The second part of the dual mechanism is required to raise the plasma calcium to the normal level of 5 meq/l and is mediated by the parathyroid glands. McLean postulates that the parathyroid hormone releases calcium from the stable crystals of hydroxyapatite, as well as from the labile fractions of bone mineral. The present work provides experimental evidence that the stable bone mineral does indeed participate in releasing calcium to the body fluids as a result of injecting parathyroid extracts. This mechanism makes an otherwise inaccessible source of calcium available for homeostatic regulation.

Summary. The effect of Parathormone injections causing mobilization of radiocalcium from incarceration in stable bone mineral of rat, has been studied by observing specific activities of bones and amounts of radiocalcium appearing in urine prior to and following subcutaneous injections of bovine parathyroid extracts. From the greatly increased renal excretion of Ca^{45} following injection of

§ The unusually high value for Group B on day 11 was probably due to inadequate washing of cage and hence incomplete collection of urine on day 10.

parathyroid extracts it can be reasonably concluded that these extracts are able to produce mobilization of stable bone mineral from the rat skeleton.

1. Singer, L., and Armstrong, W. D., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 229.

2. Singer, L., Armstrong, W. D., and Premer, M. L., *ibid.*, 1952, v80, 643.

3. Armstrong, W. D., and Schubert, J., *Anal. Chem.*, 1948, v20, 270.

4. Gley, R. O., *The Hormones,* Acad. Press, New York, 1948, vI, 271.

5. McLean, F. C., and Urist, M. R., *Bone, an Introduction to the Physiology of Skeletal Tissue,* University of Chicago Press, Chicago, 1955, p78.

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Incorporation of C^{14}-Acetate into Intestinal Fatty Acids of Rats with Cannulated Bile Ducts.* (22230)

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Incorporation of C^{14} from acetate into intestinal fatty acids has been demonstrated in experiments of short duration by various investigators(1-3). Evidence is available which indicates that the labeled intestinal fatty acids are synthesized independently of the liver fatty acids(2) and probably by the intestine itself(3,4). However, the possibility of transport of significant amounts of highly radioactive fatty acids from liver to intestine via the bile has not been investigated. This pathway was suggested to us by the findings of Gould *et al.*(5), that significant amounts of synthesized liver cholesterol are transported via the bile. The experiments described in this paper were designed to ascertain the role of bile in the acquisition of labeled long chain fatty acids by the intestine after administration of radioactive acetate.

Methods and materials. Adult male Sprague-Dawley rats weighing 200 to 300 g were used, and animals of the same weight were paired in each experiment. The bile duct of one rat was cannulated with polyethylene-50 tubing just distal to its bifurcation, and the cannula led to the outside of the body through an incision immediately below the right costal margin. A sham operation was performed in the control animal. Infection was not a problem and recovery usually occurred in 7 to 9 days. The sham-operated animal was pair-fed with the cannulated rat. During the first 2 days post-operatively there was low food consumption which led to a 5-10% decline in body weight. Subsequently, food consumption returned to normal and body weight remained at a constant level or showed a slight gain. When the rats had recovered, they were injected intraperitoneally with a tracer dose of radioactive acetate (1 ml of solution containing 3 mg of $CH_3C^{14}OO$ Na and 10 μc of activity) and placed in a cage designed to hold animals immobile(6). Water was given *ad libitum* but food was withheld after C^{14} administration. Three pairs of animals were allowed to metabolize the radioactive acetate for 5 hours; 2 pairs were killed 30 minutes after injection. Samples of bile were collected in the 5-hour experiments at 30-minute intervals for the first hour and at hourly intervals thereafter. A 0.025 ml aliquot of each bile sample was dried on a planchet and counted as such to determine the relative activity of the whole bile. In the 30-minute experiments 0.025 ml samples were taken at 5-, 15-, and 25-minute intervals. No corrections for self-absorption were applied to these measurements. The liver, intestine, bile and combined contents of cecum and intestine were taken for chemical and

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† Work done while on medical student research fellowship from National Foundation for Infantile Paralysis.

TABLE I. Specific and Total Activity of Intestinal Fatty Acids of Cannulated Rats and of Sham-Operated Controls after Administration of $CH_3C^{14}OONa$.

Time killed after inj. of C^{14}-acetate	Specific activity, c/m/mg fatty acid		Total activity, c/m/100 g rat	
	Cannulated	Control	Cannulated	Control
5 hr	109	50	3600	4600
5 "	160	109	8500	4100
5 "	73	50	4600	3500
30 min.	61	49	4700	3200
30 "	52	21	4350	1750

radioactivity analyses of the total fatty acids. Chemical analyses were done as described previously(7). To each sample of bile was added 10 mg of inert palmitic acid before saponification that adequate material might be available for analysis. Correction for this addition was made in both chemical and radioactivity analyses. For determination of radioactivity fatty acids were oxidized to carbon dioxide by the method of Thorn and Shu(8) and samples counted as barium carbonate to a total of 3000 counts in windowless flow counter.

Results. The data presented in Table I show that the total activities of intestinal fatty acids of cannulated rats were similar to those of sham-operated controls. In fact, the values obtained in cannulated animals were in most cases slightly higher than those of controls. The specific activity of intestinal fatty acids was definitely higher in the cannulated group.

Analyses of biliary fatty acids revealed that specific activity values reached a maximum in one-half to one hour after injection and remained almost constant or declined slowly thereafter. The average specific activity and total activity of biliary fatty acids for 3 cannulated rats are shown in Table II. It can be seen that in each case total activity obtained in biliary fatty acids represented only 6 to 16% of that found in the intestine itself.

TABLE II. Radioactivity of Biliary and of Intestinal Fatty Acids of Cannulated Rats after C^{14}-Administration.

Rat No.	Specific activity, c/m/mg fatty acid		Total activity, c/m	
	Biliary fatty acids	Intestinal fatty acids	Biliary fatty acids	Intestinal fatty acids
1	93	109	1600	9950
3	53	160	400	15500
5	97	72	900	12800

The specific activity of whole bile reached a maximum in one hour or less but declined at a faster rate than that of the biliary fatty acids.

The amount and radioactivity of fatty acids of combined intestinal and cecal contents were compared for normal and cannulated rats (Table III). In 4 of the 5 pairs studied the fecal material from cannulated animals contained more fatty acid than did that from controls. In 3 of the 4 experiments in which data are available the specific activity of fecal fatty acids from cannulated rats was lower than that from controls. However, total activity of the fecal fatty acids was similar for the two groups.

There was no significant effect of deviation of bile on either specific or total activity of the liver fatty acids.

Discussion. These experiments indicate that bile does not transport significant amounts of liver-biosynthesized fatty acids to the intestine. This knowledge is important in studying the possible sources of highly labeled intestinal fatty acids observed after administration of C^{14}-acetate, and the findings are consistent with the hypothesis that intestinal synthesis from acetate is the source of labeled long chain fatty acids.

A greater amount of fatty acid was found in the fecal material of cannulated rats than was found in controls. Mild steatorrhea had been observed in cannulated rats 4 to 5 days postoperatively. The increased fecal fat content was no doubt due partly to incomplete absorption of ingested fat. However, increased secretion of endogenous fat in bile-cannulated animals has also been reported (9). The lower specific activity of fecal fatty acids of cannulated rats is consistent with the presence of more fat in the intestinal lumen acting to dilute labeled fatty acids synthesized from radioactive acetate. It should be expected that increased secretion of endogenous fat in the cannulated animals would

TABLE III. Radioactivity of Fatty Acids of Combined Intestinal and Cecal Contents of Cannulated and Sham-Operated Rats after Intraperitoneal Injection of C^{14}-acetate.

Time killed after inj. of C^{14}-acetate	Total fatty acids: Amt of fatty acids, mg C*	S†	Specific activity, c/m/mg fatty acid C	S	Total activity, c/m C	S
5 hr	39.0	102.0	45.6	30.2	1780	3070
5 "	74.8	17.6	13.7	21.0	1025	370
5 "	53.0	22.6	11.8	20.3	625	460
30 min.	31.1	11.8	7.3	12.0	227	142
30 "	45.5	17.6	6.0	—	272	—

* C = Cannulated rat. † S = Sham-operated rat.

also result in increased total radioactivity in the fecal fatty acids. This was observed in 3 experiments but the opposite result was obtained in a fourth experiment.

The intestinal fatty acids of cannulated rats had a higher specific activity than those of sham-operated controls. The reason for this difference is not known.

Summary. 1. Deprivation of bile flow to the rat intestine by means of cannulation of the bile duct did not affect the ability of the intestine to accumulate normal amounts of highly labeled fatty acids after administration of C^{14}-acetate as shown by comparison with sham-operated, pair-fed controls. 2. Total radioactivity in the biliary fatty acids amounted to only 5-16% of the amount found in the intestinal fatty acids in the time periods studied. 3. Fatty acids of combined intestinal and cecal contents of cannulated animals contained more fatty acid but of a lower specific activity than those of control rats.

1. Van Bruggen, J. T., Hutchens, T. T., Claycomb, C. K., and West, E. S., *J. Biol. Chem.,* 1953, v200, 31.

2. Coniglio, J. G., and Hudson, G. W., *Fed. Proc.,* 1954, v13, 194.

3. Favarger, P., and Gerlach, J., *Helv. Physiol. Acta,* 1955, v13, 91, 96.

4. Popjak, G., and Beeckmans, M., *Biochem. J.,* 1950, v47, 233.

5. Gould, R. G., Campbell, D. J., Taylor, C. B., Kelly, F. B., Jr., Warner, I., and Davis, C. B., Jr, *Fed. Proc.,* 1951, v10, 191.

6. Bollman, J. L., *J. Lab. and Clin. Med.,* 1948, v33, 1348.

7. Coniglio, J. G., Anderson, C. E., and Robinson, C. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 666.

8. Thorn, J. A., and Shu, Ping, *Can. J. Chem.,* 1951, v29, 558.

9. Bernhard, K., and Ritzel, G., *Helv. Physiol. Acta,* 1953, v11, 166.

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Additional Serotypes of the APC Virus Group. (22231)

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Six serotypes of the adenoidal-pharyngeal-conjunctival (APC) group of viruses have been described(1,2). The purpose of this communication is to report eight additional serotypes from both human and simian sources which have been isolated in this and other laboratories.

Methods for characterization and identification of APC viruses. For purposes of classification, the cardinal attribute of the APC virus group is the group specific complement fixing antigen. This antigen is not shared with other known viruses, and complement fixing antibody against it is not produced by other known infections(1-3). The procedure presently employed for determining the presence of this antigen in candidate strains is as follows: using the complement fixation procedure previously described(2), the titer of the candidate antigen is determined by testing with a pool of human convalescent serums having high titer complement fixing antibody to the group antigen. Four units of the candidate antigen is then used in tests for antibody in paired serums of at least 4 persons, each known to have been infected with a different APC type, and each showing at least an 8-fold rise against the virus antigen

TABLE I. Neutralizing Antibody Titers of APC Prototype Rabbit Antiserums Tested against Prototype Viruses.

Rabbit antiserum	Virus types 1	2	3	4	5	6	7	8	9	10	11	D.C.	$S.V._1$	Bertha
Type 1	40	0*	0	0	0	0	0	0	0	0	0	0	0	0
2	0	40	0	0	0	0	0	0	0	0	0	0	<20†	0
3	0	0	>160	0	0	0	0	0	0	0	0	<20	"	0
4	0	0	0	320	0	0	5‡	0	0	0	0	"	"	0
5	0	0	0	0	160	0	0	0	0	0	0	0	"	0
6	0	0	5‡	0	0	80	0	0	<20	0	0	<10	"	0
7	<20	<20	<20	<20	<20	<20	320	0	"	0	0	<20	"	0
8	0	0	0	0	0	0	0	>80	"	0	0	"	0	0
9	0	0	0	0	0	0	0	0	80	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	160	0	0	0	0
11	0	0	0	0	0	0	5	0	0	0	80	0	0	0
D.C.	0	0	0	0	0	0	0	0	<10	0	0	>160	0	0
$S.V._1$	0	0	0	0	0	0	0	0	"	0	0	<20	>40	0
Bertha	0	0	0	0	0	0	0	0	"	0	0	"	0	>320

* 0 = <5. † Lowest dilution tested. ‡ Cross neutralization not observed with all rabbit serums.

of the type responsible for infection. When at least 3 of the 4 test serums show a fourfold or greater rise to the antigens of the candidate virus, the new agent can be regarded as almost certainly a representative of the APC group. Viruses are identified by the neutralization test (procedure 2) described previously(2). New serotypes are established when in reciprocal neutralizing antibody titrations with hyperimmune rabbit antiserums, the unclassified APC virus is found to be serologically distinct from all existing types. Each new prototype is examined for the presence of other properties of APC viruses, including resistance to ether, characteristic cytopathogenicity for HeLa cell cultures, trypsinized monkey kidney cultures, and explant cultures of human embryo epithelium, and lack of pathogenicity for suckling mice, adult mice, and rabbits inoculated by several routes.

Results. Fourteen serotypes have been delineated to date. Table I shows results of representative cross neutralization tests with the prototype strains. Very little cross neutralization was evident; the only heterologous neutralizations were low level, one directional crossing between Types 3 and 6, Types 7 and 11, and Types 4 and 7. Although not expressed in Table I, partial delay in cytopathogenic effects by low dilutions of heterologous antiserums was also occasionally observed(2).

The sources of the prototype strains are recorded in Table II. Three distinct types have been placed in an "incertae sedis" category. The D.C. strain grows so slowly in tissue culture that suitable comparisons with other prototype strains can be made only with great difficulty. The $S.V._1$ and Bertha strains have not been isolated from human sources and possibly should be listed as a separate simian series.

All types are ether resistant, share the group complement fixing antigen, and are nonpathogenic for suckling mice, adult mice, and rabbits. All produce similar cytopathogenic changes in cultures of monkey kidney, human embryo epithelium, and HeLa cells. In HeLa cells growth of each type results in production of excess acid in the culture fluids.

Several of the newly established types demonstrated minor differences from the previously described biological attributes of the APC virus group(2). The cytopathogenic changes produced in HeLa cell cultures by

TABLE II. Prototype Strains of APC Group.

Type	Strain designation	Isolated from	Diagnosis	Isolated by
1	Ad. 71	adenoid	hypertrophied tonsils & adenoids	NIH(2)
2	Ad. 6	"	*Idem*	"
3	G.B.	nasal washing	common cold volunteer	"
4	RI-67	throat washing	primary atypical pneumonia	Hilleman & Werner(3)
5	Ad. 75	adenoid	hypertrophied tonsils & adenoids	NIH(2)
6	Ton. 99	tonsil	*Idem*	"
7	Gomen	throat washing	pharyngitis	Berge(5)
8	Trim.	eye swab	epidemic keratoconjunctivitis	Jawetz(4)
9	Hicks	stool	arthritis, rheumatoid ?, myelitis ?	Kibrick(6)
10	J.J.	eye swab	conjunctivitis	NIH(7)
11	Slobitski	stool	paralytic polio (Type 1 polio also recovered)	Kibrick(6)
"Incertae sedis"	D.C.	anal swab	Niemann-Pick disease ?	NIH(7)
"	$S.V._1$	cynomolgous kidney	monkey kidney tissue culture	Hull(8)
"	Bertha	stool	chimpanzee with mild URI	Sabin & NIH(7)

Type 9, Type 10, and $S.V._1$ were characterized by discrete round cells throughout the cell sheet, without clumping. The $S.V._1$ strain, while cytopathogenic on primary passage, could not be maintained in serial passage in HeLa cell cultures, although it passed well in monkey kidney cultures. Large inocula of the D.C. strain were required to produce even abnormally delayed and slowly progressing cytopathogenic effects.

Several additional strains were not neutralized by antiserums to the fourteen prototypes, and these await completion of reciprocal tests before their qualifications as additional serotypes can be evaluated.

Summary. Fourteen serologically distinct APC virus types have now been established. Twelve types were recovered from human, and 2 from simian materials.

The authors are indebted to Drs. G. J. Cohen and C. Bogaert, Children's Hospital, Washington, D.C., for supplying the clinical material from which the J.J. and D.C. strains were recovered. We are also grateful to Drs. M. R. Hilleman, T. O. Berge, E. Jawetz, S. Kibrick, R. N. Hull, and A. Sabin for making strains available for comparison.

1. Huebner, R. J., Rowe, W. P., Ward, T. G., Parrott, R. H., and Bell, J. A., *New Eng. J. Med.,* 1954, v251, 1077.

2. Rowe, W. P., Huebner, R. J., Hartley, J. W., Ward, T. G., and Parrott, R. H., *Am. J. Hyg.,* 1955, v61, 197.

3. Hilleman, M. R., and Werner, J. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 183.

4. Jawetz, E., Kimura, S., Nicholas, A. N., Thygeson, P., and Hanna, L., *Science,* 1955, v122, 1190.

5. Berge, T. O., England, B., Mauris, C., Shuey, H. E., and Lennette, E. H., *Am. J. Hyg.,* 1955, v62, 283.

6. Kibrick, S., Enders, J. F., and Robbins, F. C, *J. Immunol.,* 1955, v75, 391.

7. Rowe, W. P., Huebner, R. J., Cohen, G. J., and Bogaert, C., unpublished data.

8. Hull, R. N., Minner, J. R., and Smith, J. W., to be published.

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Similarity of Glomerular Ultraviolet Absorptions in Diabetes Mellitus and After Cortisone Therapy. (22232)

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In the 20 years since Kimmelstiel and Wilson(1) described intercapillary glomerulosclerosis histologically, it has become a commonly recognized entity. Practically always, nodular glomerulosclerosis is a complication of diabetes mellitus. It is clearly not a subtype of arteriosclerosis or arteriolar nephrosclerosis(2), but little is known of the basis of its development. Experimental glomerular lesions closely simulating intercapillary glomerulosclerosis were described by Mann, Goddard and Adams(3) in rats rendered diabetic with alloxan, after 3 to 6 months, and by Rich and associates(4) in rabbits treated with 7.5 mg of cortisone daily. The pathologic process in rats was described as beginning with glomerular capillary erosions or increased deposits of periodic-acid-Schiff positive material in their walls and progressing to hyalin ball formation with concentric reticulin proliferations. Cortisone produced severe hyperglycemia and lipemia in rabbits. Recent reports by Becker *et al.*(5), Bloodworth and Hamwi(6), Wilens and Stumpf (7) confirm that cortisone therapy will lead to production of intercapillary glomerulosclerosis in normal or alloxan treated rabbits. Other factors such as vit. B_{12} deficiency, increased and altered blood lipids, and fat emboli have been reported as involved. It is uncertain how far the experimental lesions correspond to those of human diabetics, and to what extent cortisone hypersecretion might be present in human cases of glomerulosclerosis. Gordon(8) has reported unusually high levels of glycogenic adrenal steroid excretion and very low levels of 17-ketosteroid excretion in human cases of pathologically confirmed intercapillary glomerulosclerosis.

During an investigation of the histochemical and ultraviolet absorptive characteristics of kidney tissues from hypertensive patients, findings relevant to the hormonal background of diabetic glomerular lesions were unexpectedly encountered, and form the basis of this report.

Materials and methods. Freshly obtained kidney tissues from biopsies or autopsies were prepared by freeze-drying and embedded *in vacuo* in paraffin or polyethylene glycol (carbowax) with apparatus and methods previously reported(9). Microtome sections were cut at 4 μ and mounted on Vicor slides. Glycerin mounting with paraffin ringed Vicor cover slips was employed, with technics originally designed to prevent a loss of soluble substances prior to autoradiography. Ultraviolet microscopy with Polaroid color-translating instrument, was carried out in wavelength range 235-280 mμ as previously described(10). Enzyme treatment *in vitro* employed hyaluronidase in 1% solution, incubated 37°C for 30 minutes, and the solution washed off with distilled water. Treatment with trypsin, lecithinase, streptokinase and fibrinolysin was also employed, but without any significant results in the present problem. Following ultraviolet microscopy, the same sections were stained with Pearse periodic-acid-Schiff method originally introduced for staining pituitary cells(11), and studied for morphologic or histochemical abnormalities.

Results. Among hypertensive patients routinely investigated was a woman 38 years old with Cushing's syndrome, who had kidney biopsies made at the time of bilateral adrenalectomy. Each adrenal gland weighed about 40 g and was mostly composed of multiple cortical adenomas. She was not diabetic or glycosuric, and fasting blood sugar values

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TABLE I. Cortisone Therapy of 5 Cases Investigated by Ultraviolet Microscopy.

Sex	Age	Disease	Dose,† mg/day	Duration, days
♂	16	Osteogenic sarcoma	200, p.o.	30
♂	19	Hodgkin's disease	300, "	9
♀*	38	Postop. Cushing's syndrome	40, i.m.	42
♀	42	Ulcerative colitis	300, "	6
♂	42	Pulmonary fibrosis	100, p.o.	16
			75, "	2
			50, "	2
			25, "	3

* Negative observations after bilateral adrenalectomy; positive findings before operation.
† p.o. = per os; i.m. = intramuscular.

were 82, 115, 120 and 143 mg %. Ultraviolet absorptions of glomeruli and arterioles from this patient were color-translated as orange in the shortest set of wave-lengths, 248, 240 and 235 mμ, previously termed set 3.‡ This abnormality of absorption had been observed before only in diabetes mellitus(9). Three additional cases of Cushing's syndrome available for study had been previously chemically fixed in Zenker's or formalin solutions, and they failed to show any abnormality of ultraviolet absorption comparable to that of frozen-dried tissues.

Tissues prepared by freeze-drying were available from autopsies of 4 other non-diabetic persons who had had cortisone therapy until death. On testing the ultraviolet absorptive properties of their glomeruli, the same abnormal orange color of their glomerular stroma, at short ultraviolet wave-lengths, was observed. Dosages of cortisone and basic clinical information are given in Table I. The original patient with Cushing's syndrome mentioned above was maintained postoperatively on 40 mg of cortisone daily until death 42 days later. At autopsy, kidney tissues obtained and tested as previously described failed to demonstrate the abnormality observed in biopsies. This was thought to indicate that over 50 mg cortisone per day was required to induce the abnormality of glomerular stromal absorptions.

Treatment *in vitro* with hyaluronidase of kidney sections removed the material responsible for abnormal absorption. After enzyme treatment, glomerular stromal absorptions appeared normal for the range 235-280 mμ investigated. It was considered that an abnormal mucopolysaccharide component, but not necessarily hyaluronic acid, had been affected by the hyaluronidase.

Hamster kidneys were also obtained from animals treated with cortisone, approximately 6 mg per week, for use as hosts of human cancer transplants(12). These tissues also showed the abnormal orange color of translated absorptions, and the responsible material was removed by hyaluronidase. It was not possible to determine whether the animals had been diabetic.

The localization of the abnormally absorptive material in the kidneys of patients treated with cortisone differed somewhat from that previously found in diabetics(9). Orange colors of the arteriolar walls in color-translated photomicrographs appeared after cortisone, but not in diabetes mellitus. The glomerular absorptive characteristics were indistinguishable. No similar absorptive abnormality was observed in the kidneys of several persons with essential hypertension or with hypertension secondary to pheochromocytoma, being concurrently investigated.

The renal architecture of kidneys from patients treated with cortisone was not definitely altered as judged by a scrutiny of periodic-acid-Schiff stained slides. The capillary walls stained positively with the PAS technic. Occasionally a dilatation was noted of glomerular capillaries near the glomerular roots. No ectasia of peripheral glomerular capillaries, glomerular capillary aneurysms, or thrombi were observed. No lesions were found simulating intercapillary glomerulosclerosis.

Discussion. Evidence has been presented, although indirect and perhaps tenuous, implicating cortisone as one factor essential for the development of human intercapillary glomerulosclerosis. Under the influence of cortisone administered in moderate dosages the glomerular stromal tissues were altered with the accumulation of an abnormal substance

‡ Color translation by filters of 248 mμ as blue, 240 as green, and 235 as red was employed.

between the capillaries, and in their walls and those of arterioles. From the periodic-acid-Schiff staining of this material and its removal with hyaluronidase *in vitro* it was inferred to be a mucopolysaccharide complex, probably attached to protein, as usually occurs in ground substances and basement membranes.

For the formation of nodular intercapillary glomerulosclerosis in the human kidney other factors are apparently necessary, the chief of which is a part of or accompanies diabetes mellitus. Whether this influence is attributable to hyperglycemia, general changes in the body mucopolysaccharides, hyperlipemia, fat emboli, or various steroid abnormalities in human diabetics is not evident from the present study. Additional information is anticipated from the delicate lipid staining possible with carbowax embedded frozen-dried tissues. Also it is hoped that a pathologic study of the endocrine glands from diabetics with glomerulosclerosis might show some other evidences of the cortisone hypersecretion postulated.

Summary. In the glomerular stroma and arterioles of non-diabetic persons treated with cortisone an abnormal substance was found with ultraviolet absorptive properties indistinguishable from those of the nodules of intercapillary glomerulosclerosis. Histochemical properties of the material suggest that it is a mucopolysaccharide complex. The evidence is considered that implicates cortisone as one factor in the development of human diabetic intercapillary glomerulosclerosis.

1. Kimmelstiel, P., and Wilson, C., *Am. J. Path.*, 1936, v12, 83.

2. Bell, E. T., *Diabetes*, 1953, v2, 376.

3. Mann, G. V., Goddard, J. W., and Adams, L., *Am. J. Path.*, 1951, v27, 857.

4. Rich, A. R., Berthrong, M., Bennett, I. L., Jr., Cochran, T. H., Griffith, P. C., and McGoon, D. C., *Trans. Assn. Am. Physicians*, 1951, v64, 257.

5. Becker, B., Allen, R., Winter, F. C., Maengwyn-Davies, G., and Friedenwald, J. S., *Am. J. Ophth.*, 1954, v38, 53.

6. Bloodworth, J. M. B., and Hamwi, G. J., *Am. J. Path.*, 1955, v31, 167.

7. Wilens, S. L., and Stumpf, H. H., *Am. J. Path.*, 1955, v31, 275.

8. Gordon, E. S., in *Pituitary-Adrenal Function*, Ed. Ruth C. Christman, Am. Assn. Advancement Sci., Washington, D.C., 1950, pp193-201.

9. Sommers, S. C., Crozier, R., and Warren, S., *Am. J. Path.*, 1954, v30, 919.

10. ———, *Circulation*, 1955, v11, 38.

11. Pearse, A. G. E., *Stain Tech.*, 1950, v25, 95.

12. Patterson, W. B., Chute, R. N., and Sommers, S. C., *Cancer Research*, 1954, v14, 656.

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Loss and Repair of Glucose-Disposal Mechanism in Dog Fed Fructose As Sole Dietary Carbohydrate.* (22233)

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Normal rats lose, to a considerable extent, their capacity to utilize glucose when they are fed for several days a diet containing 58% fructose as *sole* carbohydrate. The site of defective glucose utilization in the fructose-fed rats was localized by studying the metabolism of C^{14}-glucose, C^{14}-fructose, and C^{14}-acetate in various tissues of rats previously fed a diet containing either glucose or fructose as sole carbohydrate. The liver was the only tissue in which an altered metabolic pattern was observed after fructose feeding, and this alteration was manifested by a decreased capacity for converting glucose to glycogen, fatty acids, and CO_2. This altered metabolic pattern in the liver was apparently not the result of an insulin-lack, for insulin injections before the fructose-fed rats were sacrificed failed to restore the ability of their livers to utilize glucose(1).

* This work was supported by contract from the U. S. Atomic Energy Commission.

TABLE I. Dietary Feeding of Each Dog.

Diet fed	Days fed	Glucose-tolerance done on:	Glucose-tolerance curve in Fig. 1
A	First 7	A.M. of 8th day	I
B	8-15	16th	II
C	16-23	24th	III
B	One meal, P.M. of 24th day	25th	IV
B	Two meals during 25th day	26th	V
D	26-32	33rd	VI

Craig *et al.*(2) failed to observe, in man, an altered tolerance to glucose following the feeding of 250 g of fructose per day (for 3 days) as the sole dietary carbohydrate. It therefore became of interest to determine whether this response to fructose was limited to the rat and also whether another hexose, galactose, would also affect the liver's capacity for utilizing glucose. Experiments designed to test these two points are reported here.

Methods. Three adult male beagle dogs (9.8, 9.2, 11.6 kg) were maintained on Purina pellets before start of experiments. Experimental feeding periods were arranged consecutively so that each dog served as its own control. The general plan of the experiment is given in Table I, and the composition of experimental diets is given in Table II. The animals' tolerance to glucose was determined in the following manner. The last portion of a specified experimental diet (A, B, C, or D) was fed at 4 p. m. on the day before the glucose-tolerance test was carried out. At 9 a. m. on the following morning, initial blood samples were withdrawn from the femoral artery, and immediately thereafter the dogs were fed a test meal consisting of 4 g glucose/kilo body weight, mixed with a few grams of ground horse meat. At hourly intervals for the next 3 or 4 hours, blood samples were withdrawn from the femoral artery. Plasma reducing value was determined by the method of Somogyi(3) as modified by Nelson(4). Plasma fructose was measured by the method of Roe(5). Plasma galactose was estimated by the difference in reducing value before and after yeast fermentation.

TABLE II. Composition of Diets.

Constituent	Amt (g)/day/kilo body wt*			
	Diet A	Diet B	Diet C	Diet D
Horse meat	47	35	35	35
Glucose	0	18	0	0
Fructose	0	0	18	0
Galactose	0	0	0	18

* Daily food allotment was divided into 2 equal portions; one portion fed at 9 a.m., the other at 4 p.m. In addition each dog received daily an adequate vitamin and salt supplement(6).

Results. Dogs fed a diet containing either no carbohydrate or glucose as sole carbohydrate. Results of the glucose-tolerance tests are given in Fig. 1. Each curve presents the average values obtained with 3 dogs. The dogs fed the diet containing no carbohydrate (diet A) for 7 days suffered the most severe loss in ability to dispose of exogenous glucose (curve I); the average plasma glucose values rose to 220 mg % 2 hours after administration of the glucose test meal and were still considerably higher than the initial level at 3 hours. In contrast, the average plasma glucose value after the dogs were fed the glucose-containing diet for 7 days reached a maximum of 120 mg % 1 hour after administration of the test glucose, and by the third hour had returned to the starting level (curve II).

Dogs fed fructose-containing diet. The average plasma glucose curve of dogs fed the diet containing fructose (diet C) for 7 days as the sole carbohydrate was similar to that obtained when the dogs were fed no carbohydrate (diet A); the highest plasma glucose value was 190 mg % at 2 hours, and the plasma glucose level was still elevated at the third hour after administration of the glucose test meal (curve III).

The feeding of one glucose-containing meal (diet B) after the 7-day period, in which fructose was the sole dietary carbohydrate, had no appreciable effect on the response of the plasma glucose to the glucose test meal, the average plasma glucose curve reaching a value of 180 mg % at 2 hours and 140 mg % at 3 hours (curve IV). However, after the third meal of diet B was fed, the average plasma glucose response was similar to that observed in the animals fed diet B for 7 days (curve V).

Dogs fed the galactose-containing diet. Feeding a diet containing galactose (diet D)

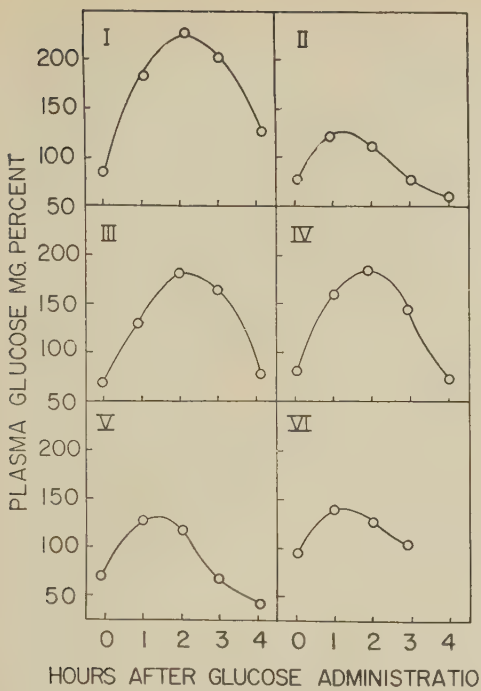

FIG. 1. Glucose tolerance tests of dogs after feeding of diets containing glucose, fructose, or galactose.

as the sole carbohydrate for 7 days produced a glycemic response to the glucose test meal similar to that observed after feeding diet B for 7 days (curve VI). In the galactose-fed dogs, the average plasma glucose level reached a maximum of 140 mg % 1 hour after administration of the glucose test meal, and returned to the initial level in 3 hours.

Summary. 1. Our earlier observation in the rat, that the feeding of fructose as sole carbohydrate results in a considerable loss in the animal's capacity to dispose of ingested glucose, is confirmed in another species, the dog. It is also shown that galactose is not the equal of fructose in bringing about impairment in glucose-disposal mechanism. 2. Feeding of a single meal containing 18 g of glucose/kg body weight failed to restore to normal the glucose-disposal mechanism once it was impaired by fructose feeding. A normal capacity for glucose disposal was observed after the dog had ingested 3 glucose-containing meals.

1. Hill, R., Baker, N., and Chaikoff, I. L., *J. Biol. Chem.*, 1954, v209, 705.

2. Craig, J. W., Miller, M., and Drucker, W. R., *J. Clin. Invest.*, 1955, v34, 928.

3. Somogyi, M., *J. Biol. Chem.*, 1945, v160, 61.

4. Nelson, N., *ibid.*, 1944, v153, 375.

5. Roe, J. H., *ibid.*, 1934, v107, 15.

6. Feinberg, H., Rubin, L., Hill, R., Entenman, C., and Chaikoff, I. L., *Science*, 1954, v120, 317.

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Attempted Fractionation of Purified Bovine Growth Hormone.* (22234)

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By any of 3 isolative procedures(1-3) growth hormone (GH) can be obtained from bovine anterior pituitary glands as an apparently homogeneous protein of molecular weight about 47,000. The activity of such preparations does not depend on the presence of alanine and phenylalanine at the N-terminal ends of the 2 peptide chains(4), nor is the activity lost after partial digestion of the free-carboxyl end of the molecule with carboxypeptidase(5-7). It may be noted also that material with growth-promoting activity can, according to an unconfirmed claim (8), pass through a dialysis membrane.

This paper reports briefly an exploration of

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the possibility that a hypothetical "active constituent" might be leached out from the bovine protein GH preparation by fractional extraction with organic solvents. The results tend to support the view of Li and his colleagues(7,9-12) that the hormonal activity is a property of the bovine GH protein as isolated, and that, if the biological activity can be associated with material of lower molecular weight, this must be obtained by partial degradation of the parent molecule.

Methods. The bovine GH preparations were isolated by the method of Wilhelmi, Fishman and Russell(2), modified by conducting the initial extractions at pH 5.5 with 0.3 M KCl, and by collecting the crude GH fraction over the ethanol concentration range 10 to 30% (v/v), beginning at pH 8.5. The purified final products had essentially unit activity (10 μg/day injected for 10 days into 100 g hypophysectomized rats produced a gain in weight of 10 g). *Bioassays.* Growth-promoting potency was determined by the 10-day test(13) in 100 g male hypophysectomized rats, obtained from Hormone Assay, Inc., Chicago. In a series of 2-dose assays, the values obtained for the response (gain in weight) in g/$\log_{10}$ μg of hormone did not vary significantly, the mean value being 16.4 (with variance 0.75). Three to 5 animals were used for each dose of standard and unknown. The 10-day incremnts in width of the tibial epiphyseal cartilage were also observed routinely. These were variable, but roughly paralleled the weight increments. *Fractional extraction of bovine GH.* The usual procedure was to treat GH with ether-miscible mixtures consisting of a "non-polar" solvent admixed with a "polar" solvent in which GH was soluble (glacial acetic acid, pyridine, phenol, formic acid, or diethyl formamide, containing a small proportion of water). Most of the mixtures contained 5% (v/v) of water, but essentially similar results were obtained in a few experiments in which the solvents were almost anhydrous. Addition of thiodiglycol or phenol as antioxidant appeared to offer no advantage. Columns 5 or 7 mm in diameter and about 3 cm in height were set up with the slurry, obtained by addition of the "non-polar" solvent (or of a mixture rich in this solvent), at –10° or 0°C, to the dry-GH admixed with 10 parts of Supercel. In subsequent operations the temperature was usually kept below 2°C. The column was percolated with the solvent mixture at a restricted rate, the mixture being replaced progressively by one richer in the more "polar" constituent if addition of ether to the effluent did not yield or no longer yielded a precipitate. The precipitates obtained from the effluents by the addition of one volume of peroxide-free ether, followed if necessary by a trace of solid potassium acetate, were centrifuged, thoroughly washed with acetone, and finally taken up in water, made slightly alkaline with sodium or ammonium hydroxide, and lyophilized. The addition of ether to the effluents left in solution only a trace of nitrogenous material which was devoid of growth-promoting activity when tested after volatilization of the solvent at a low temperature—a procedure which substantially inactivated GH in control experiments. It was found, however that volatilization of the solvent system, ammoniacal *n*-propanol-water, caused little inactivation of GH. Accordingly, this isolative procedure was employed in fractional extractions performed with aqueous propanol systems.

In several instances, fractional extractions or acetone-dried or lyophilized powders of beef anterior pituitaries and whole pig pituitaries were carried out.

Results. Experiments on purified GH were conducted with 21 different solvent systems, and on dry gland powders with 4 systems. A representative sample of potency determinations of some of the more active fractions is presented in Table I. None of the products obtained in any experiment was significantly more active than purified bovine GH. Fractional extraction typically gave poor recoveries of activity, which was usually spread over several fractions. The behavior of acetone-dried or lyophilized gland powders was similar to that of purified GH. (The ethyl-acetate-formic acid system used with the beef gland powder was particularly effective in furnishing active fractions free of pigments, these being removed in the initial stages of

TABLE I. Representative Fractional Extractions.†

Exp. No.	Material in column	Components of solvent mixture*	% (v/v) of first component	Effluent vol (ml)	Yield of product (%)	Growth potency relative to purified GH
		With 5% (v/v) water				
20	Beef ant. pituitary, acetone powder, 4 g	Acetone-acetic acid	90	74	0	—
			80	57	.5	.4
			80-70	69	9.8	.5
			60-50	81	2.3	.5
			40-30	50	4.0	.3
61	Pig pituitary acetone powder, 3 g	Ethyl-acetate-formic acid	90	115	0	—
			85	120	1.9	.1 *
			78	100	3.0	.05*
			70	70	5.4	.2 *
			63	18	6.4	.1 *
55a	Purified GH, 80 mg, with Supercell	*Idem*	90	12	10	.3
			78-65	42	0	—
			60	26	12	1.4
			55	29	33	.9
			50	15	5	.5
62a	*Idem*	Methyl-formate-formic acid	90	20	1	.2
			78	14	0	—
			70	13	5	.6
			65	21	40	.5

* Adrenals enlarged (ca. 50%) at end of test.

† Other systems used were as follows ("non-polar" constituent in italics): *ether*-phenol; -acetone-phenol-water (containing ammonia); -water (containing hydrochloric acid); -ethyl-acetate; -pyridine; -phenol-dichloroacetic acid (aqueous, 0.2%); *ethanol* (containing ammonia); *n-propanol* (containing ammonia); -phenol-ammonium formate; -ammonium flavianate; -dichloroacetic acid (aqueous, 1%); *ethyl acetate*-acetic acid; -phenol; -pyridine; *dioxane*-phenol; -pyridine. All mixtures contained water, 5%, v/v.

the extraction.) Different runs with the same system varied unpredictably in the relative yields of material obtained from successive effluents, in the ease with which the precipitates could be re-dissolved in dilute alkali, and in the stage at which flow of solvent was arrested because of gelation of the column. When successive fractions from a single run were separately re-run, characteristic differences in the extraction pattern were observed, but in no case was complete recovery of material or activity achieved. Active material was extracted from purified GH only with acidic systems. No material was extracted when the "polar" constituent was diethylformamide (with or without dichloroacetic acid), or pyridine, although GH dissolved in pyridine could be recovered in good yield and activity by precipitation with acetone. No advantage resulted from the use of GH-flavianate in place of GH itself, or from the use of ether-immiscible solvent mixtures in which the "polar" constituent was water with additions such as ammonia, ammonia and urea, ammonium formate, flavianic acid or hydrochloric acid. Finally it may be mentioned that brief dialysis of GH against a solution of 6 M urea in glycine buffer, pH 9.5, or hydrochloric acid, pH 2.0 or 3.3, gave no evidence of dialyzable activity; the remaining protein had always lost some activity. No activity was found in the "extract" obtained by treating GH with liquid ammonia.

The possibility was considered that an "active constituent" separated from GH-protein might, if it were a much smaller molecule, show poor apparent activity in the bioassay because of excessively rapid absorption from the injection site, or because of rapid excretion. In contrast with ACTH, GH is inactive when injected as a suspension in beeswax-arachis oil, but aqueous suspensions (pH 8) adsorbed on aluminum phosphate were found to be as active as aqueous solutions. In no case was the activity of fractions obtained from GH found to be enhanced by the use of aluminum phosphate.

Discussion. The experiments with dry

pituitary gland powders indicate that fractional extraction with organic solvents would offer no advantages over present methods of extracting growth hormone. In one of the best experiments, Exp. 20 (Table I), the yield of active material, 30 to 50% as potent as the purified GH used as a standard, was 34 g/kg of fresh anterior lobes, representing very likely all of the growth-promoting activity originally present. The difficulties are that the fractionation cannot be repeated consistently, and that the products are poorly soluble and are likely to prove intractable to further purification. The pig pituitary fractions were evidently rich in ACTH, as indicated by adrenal enlargement in all of the test animals. An accurate estimate of the growth-promoting properties of these pig fractions is therefore impossible to obtain. Even if these fractions were relatively rich in growth-promoting activity, the indicated order of contamination with ACTH is a decided obstacle to further purification.

It was not possible to show that an "active constituent" could be extracted from purified bovine GH preparations. Such active fractions as were obtained—by procedures which invariably depressed the solubility and inactivated the bulk of the starting material—were essentially similar to the original GH by several criteria, most significantly perhaps in being poorly soluble in water in the pH range 6-7. No differences from the original GH were evident on examination of a few of the fractions for amino-acid composition (by paper chromatography of hydrolysates), for amide nitrogen content, or for the nature and content of free amino groups (by the fluorodinitrobenzene method). It would appear, therefore, allowing the reservation that the bioassay might falsely underestimate a highly active constituent of low molecular weight, that, in agreement with Pierce(14) the biological activity is a property of the protein as isolated, and as defined by the carefully detailed studies of Li *et al.*(7,9-12).

Summary. Purified bovine growth hormone, extracted fractionally with a wide variety of organic solvent mixtures, does not yield more active constituents than the protein starting material, nor do the products differ from it in other properties.

1. Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1945, v159, 353.

2. Wilhelmi, A. E., Fishman, J. B., and Russell, J., *ibid.*, 1948, v176, 735.

3. Li, C. H., *ibid.*, 1954, v211, 555.

4. Reid, E., *J. Endocrin.*, 1952, v8, 50.

5. Condliffe, P. G., and Li, C. H., *Fed. Proc.*, 1952, v11, 198.

6. Reid, E., *J. Endocrin.*, 1953, v9, 210.

7. Harris, J. I., Li, C. H., Condliffe, P. G., and Pon, N. G., *J. Biol. Chem.*, 1954, v209, 133.

8. Dingemanse, E., and Freud, J., *Acta Brev. Neer. Physiol.*, 1935, v5, 109.

9. Li, C. H., and Pedersen, O. K., *J. Biol. Chem.*, 1953, v201, 595.

10. Li, C. H., and Ash, L., *ibid.*, 1953, v203, 419.

11. Li, C. H., and Papkoff, H., *ibid.*, 1953, v204, 391.

12. Fønss-Bech, P., and Li, C. H., *ibid.*, 1954, v207, 175.

13. Marx, W., Simpson, M. E., and Evans, H. M, *Endocrinology*, 1942, v30, 1.

14. Pierce, J. G., *Biochem. J.*, 1954, v57, 16.

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Intracellular Localization of Enzymes in *Mycobacterium tuberculosis* var *Hominis.** (22235)

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Millman and Youmans have shown that crude cell free extracts of *Mycobacterium tuberculosis* var. *hominis* H37Ra, oxidized lactic, pyruvic, acetic, fumaric, malic, succinic, citric, oxalacetic, and α ketoglutaric acids(1). This indicated that this strain of *M. tuberculosis* possessed a tricarboxylic acid terminal respiratory cycle. Millman and Youmans isolated a particulate red fraction from this extract by centrifugation at low speeds(2). This fraction, containing pleomorphic particles, oxidized fumaric, succinic, malic, oxalsuccinic, oxalacetic, α ketoglutaric, lactic, citric, and isocitric acids. A clear supernatant fraction, obtained by centrifuging the crude supernatant at 144000 x G, oxidized fumaric, malic, α ketoglutaric, lactic, and isocitric acids only in the presence of methylene blue as a hydrogen acceptor. The fact that the particulate red fraction did not require methylene blue for oxidation to occur led them to conclude that this fraction might contain a complete cytochrome system. This together with the fact that these particles reduced neotetrazolium suggested that these were identical with the structures which Mudd has called mitochondria(3). Yamamura isolated a particulate fraction from cell extracts of *Mycobacterium avium* by differential centrifugation(4). This fraction was composed of particles from 50-100 mμ diameter and was stained with Janus Green B. It was found to contain malic dehydrogenase and 3 cytochromes with bands at 554 mμ, 654 mμ and 595 mμ respectively. The author stated that this fraction behaved as a functional unit comparable to the mitochondria in animal tissue. Millman reported the isolation of a high speed sedimenting fraction composed of particles approximately 50 mμ in diameter(5). The 50 mμ particles contained some Krebs' cycle enzymes which were active in the absence of methylene blue and were cyanide sensitive. This fraction proved of greater interest when it was reported by Youmans *et al.* that it was capable of immunizing mice against challenge with a virulent strain of *Mycobacterium tuberculosis* var. *hominis*(6). Alexander and Wilson have isolated a large particulate fraction and several fractions containing smaller particles by differential centrifugation of cell-free extracts of *Azobacter vinelandii*(7). By quantitative studies they found that DPNH oxidase and p-phylenediamine oxidase occurred only in the granules, while the enzymes of the citric acid cycle were not exclusively localized in them. Isocitric dehydrogenase, α ketoglutaric dehydrogenase and aconitase were found quantitatively in the non-particulate cytoplasm. They concluded that only the particulate fractions contained an electron transport system to oxygen. In addition they found that succinic dehydrogenase and malic oxidase were totally in the particulate matter.

It is the purpose of this study to apply the methods first outlined by Alexander and Wilson(7) and Alexander(8) to a quantitative study of the distribution of enzymes in *Mycobacterium tuberculosis* var. *hominis* H37Ra.

Materials and methods. The work was carried out with the avirulent H37Ra strain of *M. tuberculosis* var. *hominis* which was maintained by frequent transfers on the surface of modified Proskauer and Beck Medium(9). Organisms were grown as surface pellicles on modified P & B medium contained in 4 liter diphtheria toxin bottles. After 25 to 35 days of incubation at 37°C the pellicles were harvested by filtration through coarse sintered glass and washed with distilled water. Approximately 25 g (dry weight) of cells were obtained from 9 bottles. The slightly moist washed cells were then made into a

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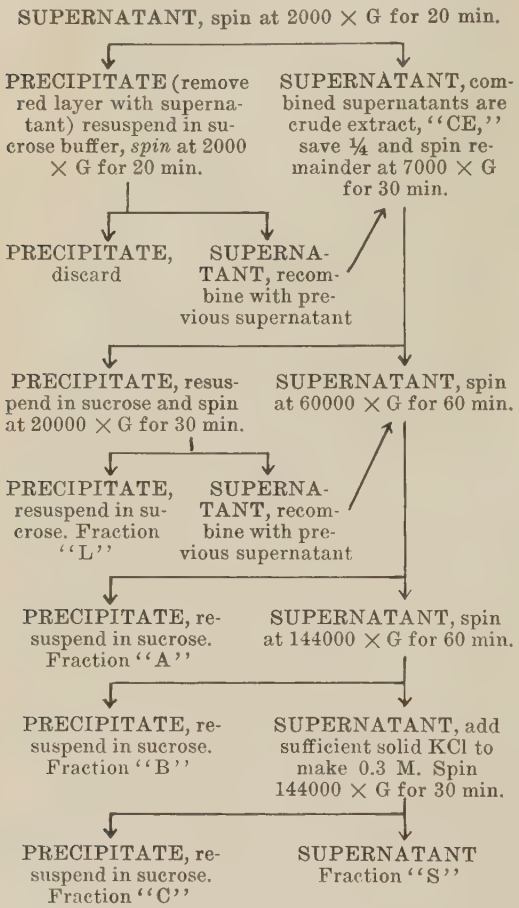
† Medical Research Fellow, National Foundation for Infantile Paralysis.

thick paste with 0.25 M sucrose dissolved in 0.01 M phosphate buffer pH 7.2 and were ground with powdered glass in a ball mill at 4°C for 18 hours. After grinding the cell mass was centrifuged at 2,000 rpm for 5 min. in an unrefrigerated International Centrifuge to remove intact cells and glass. The crude supernatant was fractionated as follows:



All precipitates were homogenized with a hand driven glass homogenizer to insure a homogeneous suspension.

Enzymatic activity. Aconitase, fumarase, malic and succinic dehydrogenases were tested by the methods described by Colowick and Kaplan(10); catalase by the method of Lawrence and Halvorson(11); DPNH oxidase by the method of Green *et al.*(12); isocitric dehydrogenase by the method of Hogeboom and Schneider(13) and α ketoglutaric dehydrogenase by the method of Lindstrom (14). Cytochrome oxidase was determined in the following way. To the main compartment of Warburg reaction vessels were added 60 μmoles of p-phenylenediamine, 16 μmoles of histidine buffer pH 7.2, 5.0 μmoles of $MgSO_4$ and enzyme preparation. To the center well of each reaction vessel were added 0.2 ml of 10% KOH. The final volume was adjusted to 2.2 ml with distilled water and oxygen uptake measured at 37°C. One unit of enzyme is that amount causing an uptake of one μl oxygen in one hour. Oxygen uptake by control flasks (containing no substrate) was subtracted from the values obtained with the test flasks before the units were calculated.

Results. Fig. 1 to 4 show electron micrographs of the particulate fractions L, A, B, and C respectively. The preparations were shadowed with chromium to help make the particles electron stable as well as aid in visualization. The L fraction is made up mainly of 100-200 mμ particles. This fraction is probably equivalent to the particulate red fraction described by Millman(5). The A fraction contains particles 50 mμ in diameter. These are probably the same as described by Millman(5) and Youmans *et al.*(6). The B and C fractions contain particles not previously described by these authors. The B fraction contains particles 25 mμ in diameter. The C fraction appears to contain particles of approximately the same dimensions as the B fraction. On close inspection, however, these particles appear as aggregates of smaller units. Because of this it has been difficult to determine the size of an individual particle. However, if this fraction is equivalent to the one reported by Alexander(8) it should contain particles approximately 19 mμ in diameter.

In Table I are summarized the results of the enzymatic localization tests. Better than 79% recovery was obtained with all but two enzymes, namely DPNH oxidase and catalase. Of the enzymes giving high recovery, aconitase, fumarase, α ketoglutaric dehydrogenase, isocitric dehydrogenase, and malic dehydrogenase were found almost exclusively in the supernatant fraction. Cytochrome oxi-

FIG. 1. Fraction L, shadowed with chromium. Contains intact tubercle bacillus and particles 100-200 mμ in diameter. × 30500.

FIG. 2. Fraction A shadowed with chromium. Contains particles approximately 50 mμ in size. × 30500.

FIG. 3. Fraction B shadowed with chromium. Contains particles approximately 25 mμ in diameter with some aggregates. × 30500.

FIG. 4. Fraction C shadowed with chromium. Contains mostly aggregates. × 30500.

dase and succinic dehydrogenase on the other hand were found mainly in the particulate fractions. Of the less completely recovered enzymes, DPNH oxidase was located almost entirely in the particulate fraction, while catalase was found mainly in the supernatant fraction. Only one enzyme showed any considerable activity in either the B or C fraction. This enzyme, fumarase, had about 12% of its total activity located in the B fraction. In relative activity the A fraction paralleled the L fraction rather closely, except that the total amount of enzyme located in the A fraction was rather less. The particulate fractions were not tested for the presence of isocitric dehydrogenase and α ketoglutaric dehydrogenase since the supernatant fraction possessed the major portion of these activities. Succinic dehydrogenase, on the other hand, gave no measureable activity in either the B, C, or S fractions.

Discussion. Our data indicate that the enzymes of the tricarboxylic acid cycle, with the exception of succinic dehydrogenase, are

TABLE I. Units of Enzyme Activity of Fractions of *Mycobacterium tuberculosis* var. *hominis* H37Ra.

Enzyme	"CE"	"L"	% CE	"A"	% CE	"B"	% CE	"C"	% CE	"S"	% CE	% recovered
Aconitase	14602	748	5.1	496	3.4	190	1.3	308	2.1	9900	67.8	79.7
Catalase	372509	1255	.33	1030	.28	4278	1.15	2653	.71	230132	61.78	64.25
Cytochrome oxidase*	4742	1697	35.8	693	14.6	173	3.6	31	.6	1175	24.8	79.4
DPNH oxidase	24105	7360	30.5	3180	13.2	348	1.4	84	.3	1100	4.6	50.0
Fumarase	58528	2550	4.4	1600	2.7	6900	11.9	820	1.4	35526	60.7	81.1
α-Ketoglutaric dehydrogenase	5006									3973	79.4	79.4
Isocitric dehydrogenase	45264									59520	131.	131. †
Malic dehydrogenase	2136000	8400	.4	33600	1.5	16800	.8	19600	.9	1620000	75.8	79.5
Succinic dehydrogenase	76	53	69.7	14	18.6							88.3

* For definition of unit, see text.

† High recovery, over 100%, probably due to partial inhibition of enzyme in "CE" by some metabolite.

either located primarily in the soluble fraction of the tubercle bacillus or in solubilized portions of the cell fragments. The presence of DPNH oxidase and cytochrome oxidase in the particulate fractions confirms the results reported in previous papers, (Millman and Youmans(1); Millman (5); Alexander and Wilson(7); and Mudd(3)) that the particulate fractions contain a complete cytochrome system and are equivalent in this respect to mitochondria of higher forms. The only metabolic difference noted between bacterial mitochondria and those of higher forms is the absence of the tricarboxylic acid cycle enzymes in the mitochondria of the former. Our results with the enzymes of the tricarboxylic acid cycle differ only slightly from those obtained by Alexander and Wilson(7). They found malic dehydrogenase in the particulate fractions while we found it in the supernatant fraction. They also found marked enzymatic activity in the B and C fractions(8) while we found negligible activity in these two fractions.

The fact that the activity of fraction A paralleled that of fraction L suggests the possibility that either the L fraction may be made up of smaller particles, or that the A fraction, and possibly the B and C fractions, may have arisen from the breakdown of the L particles.

Summary. By grinding cells of *Mycobacterium tuberculosis* var. *hominis* H37Ra using a ball mill and by differential centrifugation four particulate fractions and one supernatant fraction were obtained. Aconitase, catalase, fumarase, α ketoglutaric dehydrogenase, isocitric dehydrogenase and malic dehydrogenase were located almost entirely in the supernatant fraction, while cytochrome oxidase, DPNH oxidase, and succinic dehydrogenase were found to be associated mainly with the two largest particulate fractions.

1. Millman, I., and Youmans, G. P., *J. Bact.*, 1954, v68, 411.

2. ———, *ibid.*, 1955, v69, 320.

3. Mudd, S., *J. Histochem. Cytochem.*, 1953, v1, 248.

4. Yamamura, Y., *Symp. Enz. Chem.* (Japan), 1954, v10, 114.

5. Millman, I., *Bact. Proc. Soc. Am. Bact.*, 55th Gen. Meeting, 1955, p97.

6. Youmans, A. S., Millman, I., and Youmans, G. P., *J. Bact.*, v70, 557.

7. Alexander, M., and Wilson, P. W., *Bact. Proc. Soc. Am. Bact.*, 55th Gen. Meeting, 1955, p118.

8. Alexander, M., 1955, personal communication.

9. Youmans, G. P., and Karlson, A. G., *Am. Rev. Tuberc.*, 1947, v55, 529.

10. Colowick, S. P., and Kaplan, N. O., *Methods in*

Enzymology, 1955, v1, Academic Press Inc., New York.

11. Lawrence, N. L., and Halvorson, H. O., *J. Bact.,* 1954, v68, 334.

12. Green, D. E., Mackler, B., Repaske, R., and Mahler, H. R., *Biochem. and Biophys. Acta.,* 1954, v15, 435.

13. Hogeboom, G. H., and Schneider, W. C., *J. Biol. Chem.,* 1950, v186, 417.

14. Lindstrom, E. S., *J. Bact.,* 1953, v65, 565.

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New Technic for Study of Drug Actions on Bronchial Resistance in Isolated Lung.* (22236)

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In the estimation and evaluation of drug effects on specific organs and tissues, the use of isolated preparations perfused with a balanced salt solution has certain evident advantages. Drug and electrolyte concentration may be accurately and reproducibly controlled without the possible complications of metabolism, excretion, and binding by plasma proteins; temperature may be varied at will, and reflex and endocrine control is eliminated. Nevertheless, no completely satisfactory technic has been described for observation and measurement of drug actions on the bronchioles in an isolated lung. The object of this report is to describe an isolated lung preparation in which an attempt has been made to meet 4 basic requirements of *in vitro* technic: 1. Sensitivity to drugs should approach that shown in the intact animal. 2. Experimental conditions should simulate physiological conditions as closely as possible. 3. The method should not be encumbered with complicated apparatus and technic. 4. Results obtained should be easily interpreted and evaluated.

Most of the isolated lung preparations described in the literature have been designed for physiological studies, and technics used are rather elaborate(4-10). Usually the perfusion fluid was defibrinated or heparinised blood, which has the theoretical advantage of reducing oedema formation but introduces many new variables; it has been shown, for example, that substances may be liberated from blood which influence the tone of bronchioles(1,2). In cases where drugs were used, the sensitivity was 10-100 times lower than in the present method. Sollmon and von Oettingen(11) suggested perfusion of lungs with Locke's fluid through the bronchial tree. Although this method is very simple, it appears highly unphysiological and possesses very low sensitivity to drugs; moreover, the fluid must flow through the vascular system as well as the bronchioles, and flow rate will therefore depend on effects on the vasculature.

A large number of technics using intact animals have been described, some of which are based on principles similar to those used in the present method; *e.g.,*(12).

Method. General Principles. The object of this technic is to measure alterations in bronchial resistance in isolated lungs perfused through the pulmonary artery with a balanced salt solution. This is achieved by recording the pressure in a semiclosed system connected with the trachea while applying alternating pressure changes outside of the lungs. The recorded pressure changes will then be greatest when the bronchi and bronchioles are widely dilated, and smaller when the resistance to the passage of air between trachea and alveoli is increased. The apparatus is shown schematically in Fig. 1. It consists essentially of a warming chamber,

* The opinions or assertions contained herein are private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

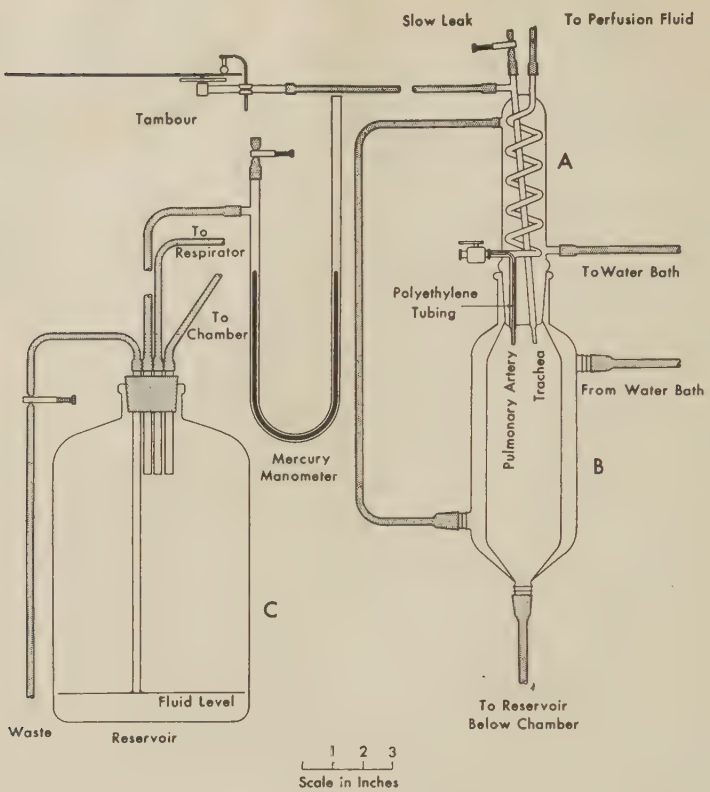

FIG. 1. Semi-schematic diagram of apparatus for lung perfusion. A is a warming chamber for tidal air and perfusion fluids; B, the lung chamber and C, the drainage reservoir, which is actually situated immediately beneath B.

A, in which the perfusion fluid and tidal air are warmed by water circulated from a thermostatic water bath at 38°C; a water jacketed lung chamber, B, in which the lungs are suspended by cannulae in the trachea and pulmonary artery from the appropriate outlets from A; and a drainage vessel C, which serves the dual purpose of collecting the fluid draining from the open pulmonary veins, and acting as a buffer between respiration pump and lung chamber to minimize alterations in applied pressure resulting from changes in volume of the lungs. A is connected with B by means of an airtight ground glass joint, and C is located immediately beneath B, to which it is attached with rubber tubing. *Perfusion* fluid is delivered to A from a Marriotte bottle, the air inlet of which is at the level of the pulmonary artery so that no positive perfusion pressure is used. Flow of fluid is maintained by pumping action of the applied air pressure changes, and is approximately 3-5 ml/min. A sidearm from the perfusion tube near the lower end of the warming vessel is plugged with a rubber stopper through which a hypodermic needle is inserted. On the inside this is connected to a polyethylene tube leading to the opening of the pulmonary artery, and on the outside a stopcock is attached. This serves to allow injections into the perfusion fluid to be made with minimum dilution and maximum speed of effect, while the stopcock prevents an air leak when the syringe is not in place. The dead space of the injection system is 0.2 ml and all injections were therefore washed in with 0.5 ml. The fluid used was a modified Krebs solution as follows: NaCl, $NaHCO_3$, KH_2PO_4, KCl, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, glucose, epinephrine in concentrations of 692, 210, 16.2, 35.4, 14.7, 18.7, 100.0 and .01 mg/100 ml, respectively. The glucose was added im-

mediately before use, and the solution was then equilibrated with 5% carbon dioxide in oxygen for at least 30 minutes. Epinephrine was added after oxygenation. The *tracheal cannula* is connected through the warming chamber with a Marey tambour recording on a kymograph. A sidearm bears a piece of rubber tubing with adjustable clamp, which during operation is kept almost closed. This slow leak serves to equalize the average pressure with atmospheric and improves the sensitivity to bronchoconstrictors. The *vessel C* is connected with the lung chamber B, respiration pump, mercury manometer and drainage tube. Total capacity of B and C is approximately 5 liters, and the respiration pump is set to move 600-800 ml of air in and out with each stroke. In a closed system the amplitude of the applied pressure change would therefore be 90-120 mm Hg. The drainage tube clamp is opened until this amplitude falls to 50-70 mm Hg, and this maintains the fluid level in C constant and at the same time equalizes the average pressure with atmospheric. Without the slow leaks on either side of the system to equalize the average pressure there was a tendency for the pressure to mount on one side and decline on the other, causing a progressive change in average volume of the lungs and in the sensitivity to drugs.

Procedure. Guinea pigs of both sexes weighed 450-550 g. The animal is given 400 units of heparin intramuscularly and 15 min. later is killed by a blow on the head. The carotid artery is immediately opened on one side, care being taken not to damage the trachea. A tracheal cannula is then inserted and artificial respiration started. The anterior wall of the thorax is opened and a polyethylene cannula tied into the common pulmonary artery through the right ventricle. The lungs are then dissected out with as little handling as possible and the heart removed. Artificial respiration is stopped and the 2 cannulae are attached to the appropriate outlets from the warming chamber A by means of adaptors. The water-jacketed vessel B is placed in position and the respiration pump started immediately. The useful life of this preparation is very dependent on the speed with which this procedure is carried out and degree of handling of the lungs. Five to 7 minutes were sufficient in our experiments.

Results. General Performance. When the preparation is first set up there ensues a progressive dilatation of the bronchioles, as evidenced by an increase in the size of the tracing, for 20-40 min. This process may be accelerated by small injections of epinephrine (2 γ), the effect of which tends to persist indefinitely (Fig. 2). During the first 60-90 min. of operation, the lungs gradually accumulate fluid until a steady state is reached, after which sensitivity to bronchoconstrictors remains approximately constant for the life of the preparation (6-8 hours). This accumulation of fluid does not interfere with movement of air, nor with response to drugs; on the contrary, the preparation does not become stable until it appears to be almost solid in the expired state.

In some preparations, however, fluid accumulated in the trachea and bronchi and persisted in spite of aspiration. This interfered grossly with movement of air and such preparations were useless for the study of drug actions. The causes of this phenomenon have not so far been determined with certainty, but certain precautions were found to reduce the incidence to a minimum and have been incorporated in the technic described. They are: 1. Addition of epinephrine to the perfusion fluid in a concentration of 1:10,000,000. In some experiments in which this was initially omitted, fluid accumulated in the trachea but recovery occurred when epinephrine was introduced and aspiration carried out. 2. Use of zero perfusion pressure. A wide range of perfusion pressures was tried, and in general the lower the pressure the less is the tendency to fluid accumulation in the trachea and bronchi. 3. Speed in dissection; avoidance of excessive handling of lungs and of inhalation of blood. Following a blow on the head, blood is readily aspirated, and rapid introduction of a tracheal cannula to avoid this is essential. 4. Premedication with heparin to prevent

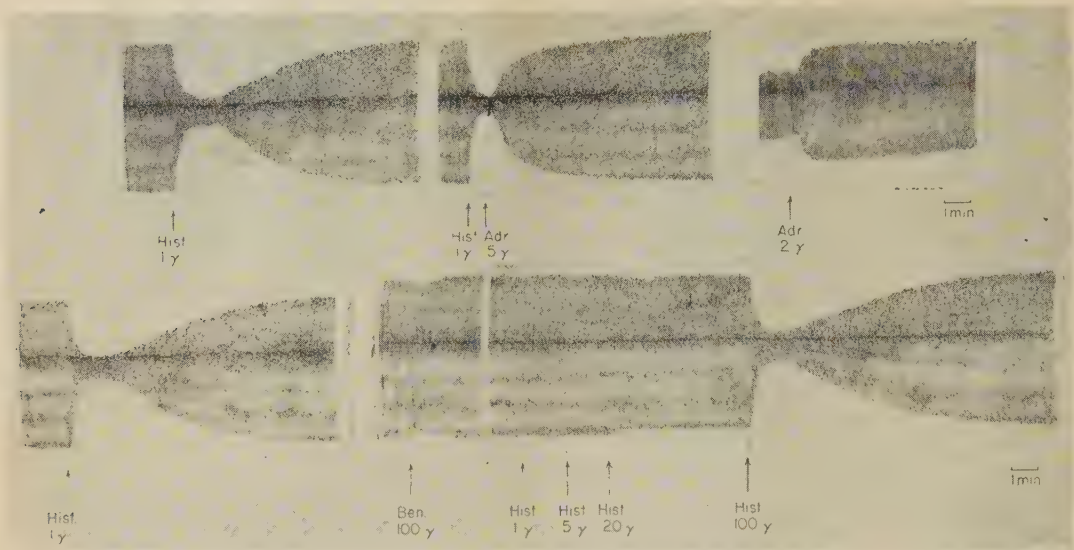

FIG. 2. Tracings showing effects of histamine, adrenaline and benadryl on the isolated lung. Tracings on top line were obtained from one preparation, those on bottom line from another. Effect of adrenaline alone is shown immediately after preparation was set up.

pulmonary emboli. 5. Positive pressure artificial respiration from the time spontaneous respiration stops until the preparation is finally set up. 6. Use of young animals. If the animal is too small, however, the tracing obtained is too small to work with; 450-500 g guinea pigs were found to be a suitable compromise.

Response to histamine. The injection of 1 γ histamine diphosphate produces a rapid reduction in amplitude of the tracing, indicating bronchoconstriction. The effect persists for a little over a minute and complete recovery ensues in 6-8 min. (Fig. 2, top line). Perfusion rate is almost unchanged, a 0-10% reduction in outflow being seen. This response is reproducible for several hours provided that the conditions are maintained constant. However, temporary mechanical disturbance of perfusion flow may cause a longer lasting change in sensitivity to histamine. It is therefore advisable for assay purposes to use a Marriotte bottle large enough to last for the entire experiment without refilling. An increase in sensitivity up to 10-fold may be achieved by omitting epinephrine from the perfusion fluid; however, there is then a greater tendency for fluid accumulation in the trachea, and recovery from histamine is slower. Sensitivity to histamine may be reduced very greatly by antihistamines. Fig. 2 (lower tracing) shows a sequence taken from an experiment in which a single injection of 100 γ of benadryl reduced the sensitivity to histamine by a factor of approximately 100.

Response to other agents. Acetylcholine produces a response closely resembling that of histamine, with slightly faster recovery, at

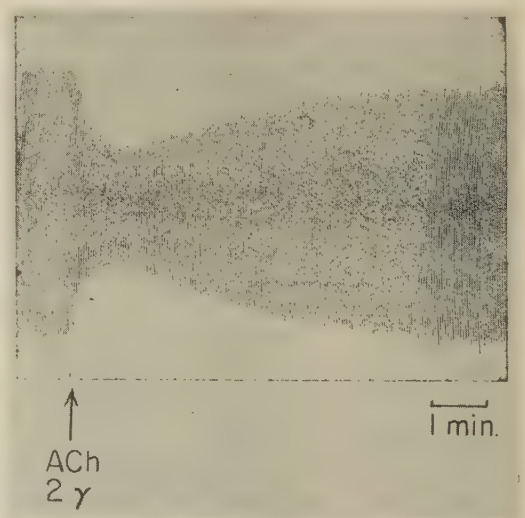

FIG. 3. Tracing showing effects of acetylcholine on isolated lung.

about 3 times the dose (Fig. 3). This response may be blocked by atropine. Morphine sulphate produces no bronchoconstriction in doses of up to 250 γ.

Bronchodilators have little or no action on the standard preparation, presumably because the bronchi are maintained maximally dilated by the epinephrine. Even epinephrine, in doses up to 5 γ, produces only a slight and very temporary effect. When superimposed on a typical response to histamine, however, epinephrine has a marked effect in accelerating recovery (Fig. 2); papaverine and theophylline also show some dilator activity in rather large doses (250 γ and 500 γ respectively). As mentioned earlier, epinephrine also produces a well marked effect on a preparation newly set up, and if epinephrine is omitted from the perfusion fluid, marked sensitivity is shown to bronchodilators.

Summary and Conclusions. A technic for the observation and measurement of the effects of drugs on bronchial resistance in an isolated perfused lung preparation is described. This preparation shows an absolute sensitivity to histamine comparable to commonly used biological assay objects, and shows a reproducibility adequate for assay purposes. Its simplicity makes it suitable also for teaching demonstrations of the effect of drugs on the bronchioles.

1. Bayliss, L. E., and Ogden, E., *J. Physiol.,* 1933, v77, 34P.

2. Zucker, M. B., *Am. J. Physiol.,* 1944, v142, 12.

3. Gaddum, J. H., Hebb, C. O., Silver, Ann and Swan, A. A. B., *Quart. J. Exp. Physiol.,* 1953, v38, 255.

4. Daly, I. deB., *J. Physiol.,* 1928, v65, 422.

5. ———, *ibid.,* 1930, v69, 238.

6. Berry, J. L., and Daly, I. deB., *Proc. Roy. Soc.,* 1931, v109B, 319.

7. Alcock, P., Berry, J. L., and Daly, I. deB., *Quart. J. Exp. Physiol.,* 1936-7, v26, 13.

8. Dale, A. S., and Narayana, P., *ibid.,* 1935, v25, 85.

9. Binet, L., and Bargeton, D., *J. Physiol. Path. Gen.,* 1939-40, v37, 1217.

10. Nisell, O. I., *Acta Physiol. Scand.,* 1950, v21, Supp. 73.

11. Sollmann, T. and von Oettingen, W. F., PROC. SOC. EXP. BIOL. AND MED., 1927, v25, 692.

12. Seibert, R. A., and Handley, C. A., *J. Pharmacol.,* 1954, v110, 304.

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Potentiation of Glycogenolytic Effect of Epinephrine by Desoxycorticosterone in Certain Skeletal Muscles.* (22237)

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The glycogenolytic effect of epinephrine on skeletal muscle (hind limb) is inhibited by cortisone acetate in normal rats(1), by adrenal cortical extract in adrenalectomized rats(2), and by growth hormone in hypophysectomized rats(3). On the other hand, adrenalectomy(2,4) or the injection of thyroxin(3,4) potentiates the glycogenolytic action of epinephrine and thyroidectomy depresses it(4). Those hormones which inhibit this effect of epinephrine also induce a storage of glycogen in these skeletal muscles (glycopexic effect)(1,2,3,5). While it is usually stated that desoxycorticosterone does not stimulate glycogen storage in muscle(5-8), there is no information on whether this mineral-corticoid influences epinephrine effects on carbohydrate metabolism in the intact animal. This report will show that desoxycorticosterone acetate (DOCA) potentiates the glycogenolytic action of epinephrine in certain skeletal muscles.

Materials and methods. Adult female rats of the Long-Evans strain were selected for uniformity of age and weight within each ex-

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periment. The animals were fed a diet consisting of calf meal and dog pellets *ad libitum*. The adrenalectomized animals received 1% salt solution in addition to drinking water. Subcutaneous injections of 2 mg DOCA† per day in peanut oil were administered for 3 days prior to autopsy. Hormone treatment was begun one week after adrenalectomy and 3 weeks after thyroidectomy. Epinephrine was injected intraperitoneally in doses of 10 γ per 100 g body weight one hour prior to autopsy. In all experiments the rats were starved for 24 hours prior to the administration of epinephrine. The muscles used in this study were the *rectus femoris*, a portion of the abdominal muscles and the diaphragm. The method for anesthetizing the animals, removing the muscles, and determining glycogen has been previously described(9). The glycogen is reported as mg of glucose per 100 g of muscle, wet weight (averages with the standard error of the mean).

Results. The injection of DOCA into normal intact rats had no significant effect on the glycogen content of any of the muscles used in this study (Table I-A), which confirms earlier work(5-8). The injection of epinephrine into the normal rats decreased the glycogen content in all 3 muscles significantly. When the rats were pretreated with DOCA, however, epinephrine induced a greater loss of glycogen in the leg and abdominal muscles as compared to the loss observed in the untreated rats. There was no potentiation of the epinephrine effect in the diaphragm. If one compares the loss of glycogen expressed as a percentage of the respective initial levels in the DOCA-treated and control animals, it is noted that the greatest potentiation of epinephrine occurred in the leg muscle (44% *vs* 23% loss), with an intermediate response in the abdominal muscle (62% *vs* 46%).

In view of the previous finding that glucocorticoids inhibited the glycogenolytic action of epinephrine in some muscles(1,2), it was considered possible that the administration of DOCA suppressed the endogenous secretion of the gluco-corticoids via the pituitary(10) which would essentially remove an inhibitor to the action of epinephrine. To test this possibility, the effect of DOCA was studied in adrenalectomized rats.

The data in Table I-B show that adrenalectomy alone had no significant effect on the glycogen levels of the 3 muscles when compared to the intact controls. Following the administration of epinephrine, a significant decrease in muscle glycogen was exhibited by all 3 muscles. Furthermore, the loss of glycogen was greater in the adrenalectomized rats than in the intact rats but only in the leg muscle. Pretreatment of the adrenalectomized rats with DOCA caused a significant increase in glycogenolysis only in the leg muscle.

Collip *et al.*(4) and Leonard(3) have reported that thyroxin potentiates the glycogenolytic action of epinephrine in hypophysectomized animals. This finding introduces the possibility that the potentiating effect of DOCA might be conditioned by the thyroid hormone: accordingly, the effect of DOCA on glycogenolysis was investigated using thyroidectomized rats.

The data show (Table I-C) that thyroidectomy alone causes no appreciable decrease in muscle glycogen. Administration of epinephrine to the thyroidectomized rats caused no significant decrease in the glycogen levels of the *rectus femoris* or abdominal muscles; the diaphragm exhibited a significant decrease. Pretreatment of the thyroidectomized animals with DOCA failed to increase the glycogenolysis induced by epinephrine.

Progesterone is reported to have properties similar to DOCA in regard to salt and water retention(11) and also to its inability to stimulate the deposition of glycogen in muscle(12,13). Accordingly, an experiment was made to determine whether progesterone, like DOCA, would potentiate the glycogenolytic action of epinephrine.

The data in Table I-D show that intact rats pretreated with 2 mg progesterone per day for 3 days exhibited no significant increase in glycogenolysis above that obtained with epinephrine alone.

† Desoxycorticosterone acetate and progesterone were donated by Schering Corp., Bloomfield, N. J.

TABLE I. Effect of DOCA and Progesterone on Epinephrine Induced Glycogenolysis.

Exp.	No. of rats	Glucose (mg/100 g muscle) *Rectus femoris*	Abdominal	Diaphragm
Part A—Intact rats				
Control	16	333 ± 13	428 ± 11	285 ± 7
DOCA treated	6	321 ± 15	424 ± 17	306 ± 11
E† treated	15	256 ± 6	233 ± 10	107 ± 8
E & DOCA treated	10	178 ± 9*	160 ± 14*	111 ± 9
Part B—Adrenalectomized rats				
Control	10	339 ± 10	377 ± 12	313 ± 17
E treated	15	228 ± 5	203 ± 9	122 ± 8
E & DOCA treated	14	169 ± 9*	183 ± 10	96 ± 8
Part C—Thyroidectomized rats				
Control	4	309 ± 27	377 ± 21	269 ± 13
E treated	12	293 ± 15	281 ± 26	122 ± 16
E & DOCA treated	12	269 ± 17	291 ± 22	132 ± 20
Part D—Intact rats				
P† treated	5	352 ± 10	430 ± 13	246 ± 21
P & E treated	10	251 ± 9	198 ± 13	86 ± 21

* In all cases where DOCA increased glycogenolytic effect of epinephrine significantly ($P < .01$), results are marked with an *.

† E = Epinephrine; P = Progesterone.

Discussion. These results show that the mineral-corticoid, DOCA, in some manner enhances the glycogenolytic action of injected epinephrine in the *rectus femoris* and the abdominal muscles, but not in the diaphragm. In contrast, the gluco-corticoid, cortisone, inhibited this effect of epinephrine to the greatest extent in the leg muscle, less in the abdominal muscles and not at all in the diaphragm(1). There is a basic difference in sensitivity to epinephrine among these three muscles in the untreated rat; the diaphragm is the most sensitive and the *rectus femoris* the least.

The possibility that DOCA was repressing the secretion of the adrenal gluco-corticoids via pituitary ACTH, thus removing an inhibitor to the action of epinephrine, was discounted when it was observed that DOCA potentiated glycogenolysis in the adrenalectomized animal. This suggests that DOCA is acting directly on the muscle to potentiate epinephrine.

At first sight it appeared that some interrelationship with the thyroid gland was involved, since DOCA did not potentiate epinephrine in the thyroidectomized animal. However, the loss of thyroid activity in an animal is known to decrease the absorption of injected epinephrine(14) and this decrease could be of such magnitude that any effect of DOCA would be unobservable. It is reported that DOCA increases the absorption of electrolytes from the intestine in adrenalectomized rats(15) and increases the permeability of the skin(16). If DOCA, in potentiating epinephrine, acts by increasing the permeability of the target organ to the passage of epinephrine, adequate thyroid secretion may be essential for this effect. It seems more likely that thyroidectomy acts by preventing absorption of the epinephrine at the injection site.

A concept of mutual antagonism between the 2 types of adrenal cortical steroids on certain stress reactions and on the specificity of their antagonism to definite target organs has recently been discussed by Selye(17). It may be that the normal maintenance and regulation of glycogen storage in skeletal muscle is dependent in part on the relative endogenous production of the two types of adreno-corticoids and epinephrine. There is little evidence at present to indicate how much the normal output of epinephrine affects the level of muscle glycogen in the intact animal. Bowman(18) has shown that in the hypophysectomized animals which lack the glycopexic hormones, removal of the whole adrenal gland induces a rise in the glycogen level in fasting rats. The increase in muscle glycogen would seem to be caused by the elimination of endogenous epinephrine.

Finally, it was observed that progesterone was unable to potentiate the glycogenolytic action of epinephrine.

Summary. Desoxycorticosterone acetate increased the glycogenolytic action of epinephrine in the *rectus femoris* and abdominal muscles but not in the diaphragm in intact rats. In adrenalectomized rats, the effect was limited to the *rectus femoris*. Thyroidectomy decreased epinephrine induced glycogenolysis, and DOCA was unable to nullify this inhibi-

tory effect. Progesterone had no effect on the glycogenolytic action of epinephrine.

1. Wortman, L. C., and Leonard, S. L., *Endocrinology,* 1953, v53, 480.
2. Winternitz, W. W., and Long, C. N. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 683.
3. Leonard, S. L., and Ringler, I., *Endocrinology,* 1954, v55, 212.
4. Collip, J. B., Thomson, D. L., and Toby, G., *J. Physiol.,* 1936, v88, 191.
5. Leonard, S. L., *Endocrinology,* 1953, v53, 226.
6. Long, C. N. H., Katzin, B., and Fry, E. G., *ibid.,* 1940, v26, 309.
7. Reinecke, R. M., and Kendall, E. C., *ibid.,* 1943, v32, 505.
8. Winter, C. A., and Thomson, J. D., PROC. SOC. EXP. BIOL. AND MED., 1944, v55, 95.
9. Leonard, S. L., *Endocrinology,* 1952, v51, 293.
10. Cheng, C., and Sayers, G., *ibid.,* 1949, v44, 400.
11. Thorn, G. W., and Harrop, G. A., *Science,* 1937, v86, 40.
12. Corey, E. L., *Am. J. Physiol.,* 1941, v132, 446.
13. Unpublished data from this laboratory.
14. Russell, J. A., and Cori, G. T., *ibid.,* 1937, v119, 167.
15. Stein, L., and Wertheimer, E., PROC. SOC. EXP. BIOL. AND MED., 1942, v46, 172.
16. Opsahl, J. C., *Yale J. Biol. and Med.,* 1948, v21, 255.
17. Selye, H., *Science,* 1955, v122, 625.
18. Bowman, R. H., Doctoral Thesis, Cornell University, 1952.

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Inhibition Studies with *Streptococcus faecalis.* Acridines and Acridones. (22238)

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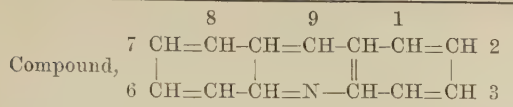
The investigations of Woods(1) led to the finding that the sulfonamides owed their chemotherapeutic activity to their property of interfering with the utilization of p-aminobenzoic acid by the microbial cell. This observation has stimulated the synthesis and testing of compounds related closely in structure to known metabolites for trial in the controlled interference with selected metabolic reactions. In the instance of folic acid, this has given rise to a series of folic acid blocking agents(2), some of which have proven usefulness(3), though limited, in a variety of ways. The interference with the utilization of folic acid was applied by Hitchings, among others, to develop compounds of biological interest. As a result Hitchings and his associates observed(4,5) that the rather active anti-folic, pyrimethamine, appeared also to possess some promise against the malarial parasite, though continued study may have modified this view somewhat(6,7).

In view of the findings that compounds which interfere with the utilization of folic acid may have application in other disorders for which agents are needed, a reinvestigation of 53 available acridines(8,9) and acridones (10), prepared initially for testing against the malarial parasite, was undertaken.

Methods. The folic acid assay medium utilized in this study was slightly modified from that of Teply and Elvehjem(11). The modifications consisted of omitting the xanthine and of utilizing "vitamin free" casein hydrolysate (Nutritional Biochemicals) in place of the charcoal treated hydrolysate originally suggested. When folic acid was added in amounts up to 1.0 mμg per ml of medium the acid production upon titration with N/10 alkali went up from blanks of about 0.5 ml of alkali per tube to 9.5 to 10 ml of alkali per tube at the higher levels of added folic acid. The test compounds were added to tubes containing 0.32 mμg of folic acid per ml of medium, a level designed to support half maximum growth as judged by acid production. This usually ranged about 4.5 to 5.0 ml of N/10 alkali per tube. For the assays, the test compounds were added to duplicate tubes usually at 4 levels of test: 1.6, 8,

TABLE I. Acridines Which Interfere with Utilization of Folic Acid by *S. faecalis*.

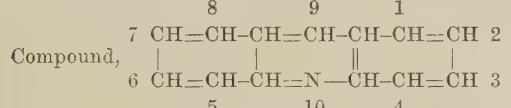


Substituent at 9	Derivatives	Activity vs. folic acid, μg/ml
9-NH_2 (aminacrine)	4-CH_3	8/ 40
9-$NH(CH_2)_3NHCH_2CH_2OH$	2-OCH_3	40/200
" $NHCH_2CHOHCH_3$	"	"
" $NH(CH_2)_2CHOHCH_3$	"	"
9-$NH(CH_2)_4N(CH_3)_2$	2-OCH_3 6-Cl	"
" $N(C_2H_5)_2$	"	"
9-$SCH(CH_3)(CH_2)_3N(C_2H_5)_2$	"	"
9-$NH(CH_2)_3S(CH_2)_2N(C_2H_5)_2$	"	"
9-NH $C_6H_4N(CH_3)_2$	"	"

40, and 200 μg per ml of final medium. The tubes were plugged, sterilized about 12 minutes at 120°C, cooled at room temperature, and inoculated(12), with a drop of a suspension of *Streptococcus faecalis* (A.T.C.C. No. 8043). The growth of the organism was estimated titrimetrically after 72 hours using brom thymol blue as the indicator.

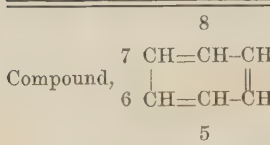
Results. The acridines and acridones tested and their ability to interfere with the utilization of limiting levels of folic acid in the medium are summarized in Tables I-III. To characterize the inhibitory activity of the compounds, in instances where inhibitory activity was observed, an arbitrary ratio term has been used. The upper (and lesser) figure represents the amount of test compound which exercised little or no inhibition of acid

TABLE II. Acridines Which Do Not Interfere with Utilization of Folic Acid by *S. faecalis*.



Substituent at 9	Derivatives
9-NH_2	2-OCH_3 6-Cl, 3-NO_2 6,7-$(OCH_2)_2$
9-$NHCH_2CH_2OH$	3-NO_2 6,7-$(OCH_2)_2$
9-$NHCH_2CH_2NH_2$	2-OCH_3 6-Cl
9-(3′-C_5H_4N)	"
9-(6′-OCH_3-8′-quinolyl-NH_2)	"
9-$NH(CH_2)_2NH(CH_2)_2CH_2OH$	" (unsub.), 6-NO_2 2,3-$(OCH_3)_2$
" $NHCH_2CHOHCH_3$	2-OCH_3, 2-OCH_3 6-Cl, 2,3-$(OCH_3)_2$ 6-Cl
" $NHCH_2CHOHCH_2OH$	2-OCH_3, 6-Cl, 2-OCH_3 6-Cl
" $NHCH_2C(OH)(CH_3)_2$	2-OCH_3, 2-OCH_3 6-Cl
9-$S(CH_2)_2N(C_2H_5)_2$	2-OCH_3 6-Cl
9-$O(CH_2)_2N(C_2H_5)_2$	"
9-$NH(CH_2)_3NHCH_2CH_2OH$	"
" $NHCH_2CHOH\ CH_3$	"
" $NH(CH_2)_2CHOH\ CH_3$	"
" $NHCH_2C(OH)(CH_3)_2$	2,3-$(OCH_3)_2$ 6-NO_2
" $N(C_2H_5)CH_2CHOH$	2-OCH_3 6-Cl
9-$NHCH_2CHOHCH_2N(C_2H_5)_2$	2,3-$(OCH_3)_2$6-NO_2 (entozon)
9-$NHCH(CH_3)(CH_2)_3N(C_2H_5)_2$	2-OH 6-Cl, 2-OCH_3 6-Cl (quinacrine), 2,3-$(OCH_3)_2$ 6-Cl, 2-OCH_3 6-CH_3, 2-F 6-Cl
9-$NHC_6H_4N(CH_3)_2$	
9-$NHCH_2CH(C_6H_5)(CH_2)_2N(CH_3)_2$	2-OCH_3 6-Cl
" $(CH_2)_2N(C_2H_5)_2$	"
9-$NHCH_2(C_6H_5)C_5H_8NCH_3$	"
" $C_5H_8N\ CH_2C_6H_5$	"

TABLE III. Effect of Acridones on Utilization of Folic Acid by *S. faecalis*.



Compound, Substituent at 1	Derivatives	Activity vs. folic acid, μg/ml
1-$NH(CH_2)_2N(C_2H_5)_2$	4-CH_3, 4-$CH_3$6-Cl	None
1-$NH(CH_2)_3N(C_2H_5)_2$	3-Cl, 4,6-Cl_2	8/ 40
"	4-Cl	40/200
1-$NHCH_2CHOHCH_2N(C_2H_5)_2$	4,6-Cl_2	"
1-$NHCH(CH_3)$ $(CH_2)_3N(C_2H_5)_2$	4-Cl	"

production (growth) of the organism. The lower (and larger) value represents the amount of test substance which appreciably or completely inhibited acid production by the organism. This method of evaluating the test compounds has proven more reproducible than the calculation of the 50% molar inhibition index. The latter has been observed to vary to a disturbing extent following small changes in the test conditions, whereas the ratio term has remained constant in the face of minor modifications in the testing of the compounds.

The results summarized in Tables I-III probably may be rendered more meaningful if compared with those obtained under these conditions of test on some known effective anti-folics. The inhibition ratios against folic acid and citrovorum factor, respectively, were: aminopterin 0.008/0.04 and 0.2/1.0, amethopterin 0.00032/0.0016 and 0.04/0.2, and pyrimethamine 0.0016/0.008 and 25/100.

From the data of Tables I and III it may be seen that 40 to 200 μg of 10 acridines and of 5 acridones per ml of medium proved to be inhibitory. These amounts were 1000-fold (or more) those needed by the anti-folics given above. The acridine and acridone inhibitors thus appear to be primarily of theoretical interest at this time. The activities of the inhibitory acridines and the acridones were further differentiated. With the same organism and with 2.0 mμg of citrovorum factor(13) per ml of medium as the limiting nutrient 6-chloro-9(4-diethylamino-1 methylbutylthio)-2-methoxy acridine and 1-(3-diethylamino-2-hydroxypropylamino)-4, 6-dichloroacridone were no longer inhibitory. In the presence of 2.5 μg of thymine(14,15) per ml of medium and with *S. faecalis* as the test organism, 9-[3-(3-hydroxybutylamino)propylamino]-2-methoxyacridine no longer inhibited. In the presence of 2.5 μg of thymidine(16) and with *S. faecalis* as above, 2-methoxy-6-chloro-9 - (4′-dimethylaminophenylamino)-acridine, 6-chloro-2-methoxy-9-(4-dimethylaminobutylamino)acridine, and 9-[3-(2-hydroxyethylamino) propylamino] - 2-methoxyacridine no longer inhibited the organism.

The remaining 5 acridines of Table I (9-aminoacridine, 9-amino-4-methylacridine, 2-methoxy-6-chloro - 9(4 - diethylaminobutylamino)propylamino)-acridine, 9-[3-(2-hydroxypropylamino]-2-methoxy acridine, 6-chloro-2-methoxy-9(γ-(β′-diethylaminoethylthio)-propylamino) acridine, and 4 of the acridones of Table III (3-chloro-1-(3-diethylaminopropylamino)-acridone, 4-chloro-1-(3-diethylaminopropylamino)-acridone, 4, 6-dichloro-1 - (3-diethylaminopropylamino)-acridone, 4-chloro-1-(4-diethylamino-1-methylbutylamino)-acridone) which inhibited *S. faecalis* in the presence of limiting amounts of folic acid were inhibitory whichever of the above nutritiles were limiting in the medium. Whether the inhibitory activity of these compounds was due to interference in the metabolic sequence of nucleic acid metabolism or whether it was due to blocking some other metabolic sequence is not known at this time.

Another point of interest arises from these studies. It may be noted that the effective antimalarial, quinacrine (mepacrine), does not

inhibit the organism in the presence of limiting amounts of folic acid. Parenthetically, a number of salts of quinacrine were tested so that the results are not limited to one salt. Thus, the acridine anti-malarials appear to owe their anti-malarial activity to some property other than interference in nucleic acid metabolism. This must be qualified, however, for the reason that it is not proven that results with *S. faecalis* may be carried over *in toto* to the malarial parasite.

Like quinacrine, the anti-bacterial Entozon (nitroacridine 3582, Hoechst) did not inhibit the test organism under the conditions of the test with folic acid as the limiting nutrilite. Contrariwise, the anti-bacterial 5-aminoacridine (aminacrine) inhibited the organism whichever nutrilite was present in limiting concentrations.

Thus, the studies with *S. faecalis* demonstrate interesting selectivity with various acridines and acridones. Whether they will remain of theoretical interest only remains to be determined.

Summary. 1. 46 Acridines and 7 acridones were tested against *Streptococcus faecalis* in the presence of limiting amounts of folic acid. It was observed that 10 of the acridines and 5 of the acridones inhibited the organism. However, the amounts of test compounds needed were 1000 times (or more) those needed by aminopterin, amethopterin, and pyrimethamine. 2. Further study demonstrated that inhibition exhibited by acridines and acridones could be overcome by citrovorum factor, thymine, or thymidine in some instances. The toxicity of 5 of the acridines and 4 of the acridones was not overcome by any of the nutrilites tested which suggests that their inhibition was not due to interference with the nucleic acid metabolism of the organism. 3. An additional observation was that the anti-malarial quinacrine and the anti-bacterial Entozon did not inhibit *S. faecalis* in the presence of limiting amounts of folic acid. The anti-bacterial 5-aminoacridine was inhibitory whether the limiting nutrilite was folic acid, citrovorum factor, thymine, or thymidine.

1. Woods, D. D., *Brit. J. Exp. Path.,* 1940, v21, 74.

2. Jukes, T. H., Franklin, A. L., and Stokstad, E. L. R., *Ann. N. Y. Acad. Sci.,* 1950, v52, 1336.

3. Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., and Wolff, J. A., *N. Engl. J. Med.,* 1948, v238, 787.

4. Hitchings, G. H., Elion, G. B., Falco, E. A., Russell, P. B., Sherwood, M. B., and Vander Werff, H., *J. Biol. Chem.,* 1950, v183, 1.

5. Hitchings, G. H., Elion, G. B., Falco, E. A., Russell, P. B., and VanderWerff, H., *Ann. N. Y. Acad. Sci.,* 1950, v52, 1318.

6. Dennis, E. W., and Berberian, D. A., *Ann. Rev. Med.,* 1953, v4, 345.

7. Murphy, A., Ellison, R. R., Karnofsky, D. A., and Burchenal, J. H., *J. Clin. Invest.,* 1954, v33, 1388.

8. Surrey, A. R., Suter, C. M., and Buck, J. S., *J. Am. Chem. Soc.,* 1952, v74, 4102.

9. Albert, A., *The Acridines,* E. Arnold and Co., London, 1951.

10. Archer, S., Rochester, L., and Jackman, M., *J. Am. Chem. Soc.,* 1954, v76, 588.

11. Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.,* 1945, v157, 303.

12. Black, T. L., and Arnold, A., *Ind. Eng. Chem., Anal. Ed.,* 1940, v12, 344.

13. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.,* 1948, v176, 165.

14. Stokes, J. L., *J. Bact.,* 1944, v48, 201.

15. Hutchings, B. L., Mowat, J. E., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and Subbarow, Y., *J. Biol. Chem.,* 1947, v170, 323.

16. Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.,* 1948, v70, 2299.

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B M R and Thyroxine Secretion Rate in Salivariectomized Female Rats.* (22239)

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The salivary glands have been known for many years to play a role in iodine metabolism by secreting (excreting?) blood iodide as a component of saliva. Recently a function of the salivary glands in iodine metabolism has been reported by Fawcett and Kirkwood(1,2) in the rat, namely to deiodinate diiodotyrosine and monoiodotyrosine, the latter by virtue of the presence of an enzyme, tyrosine iodinase. These workers also reported that the rapid deiodination of intravenously injected diiodotyrosine which occurs in normal rats was prevented (up to 60 minutes) in rats previously salivariectomized. Consequently the suggestion has been made (1) that not only is the blood level of thyroxine in the rat maintained by the rate of thyroxine synthesis in the thyroid gland but also by the rate of destruction of thyroxine and/or its precursors, monoiodotyrosine and diiodotyrosine wherever this may occur.

The purpose of the experiments reported herein was to determine the effect of salivariectomy on thyroid function as shown by the BMR and thyroxine secretion rate; *viz.* do the salivary glands play an essential role in extrathyroidal thyroxine metabolism or can their function be dispensed with without seriously disturbing thyroxine metabolism?

Procedure. Young adult Sprague-Dawley female rats, fed Purina Laboratory Chow and water *ad lib.*, were used in these 2 experiments. In the *first experiment,* 8 female rats were salivariectomized under pentabarbital (Nembutal) anesthesia and allowed to recover; 8 other normal females served as control animals. The individual body weight range for these 16 animals was 100-125 g. The BMR's of these rats were determined from O_2 consumption data obtained by using a closed, large glass desiccator with a water manometer and an attached 10 cc syringe filled with air. CO_2 was absorbed by NaOH (Ascarite). The pressure inside the desiccator was kept relatively constant by replacing periodically the oxygen consumed with air from the syringe. Constant temperature was maintained by use of a water bath at room temperature. The BMR's used in the final calculation for the individual rats were averages of at least 2 determinations. In the *second experiment,* thyroxine secretion rates of 23 salivariectomized and 25 intact rats were calculated by comparing thyroid gland weights at autopsy of a control group of rats having received no thiouracil with 3 groups of animals having received .1% thiouracil† in their drinking water for 2 weeks; in addition, 2 of the latter 3 groups were injected daily with 3 γ and 6 γ respectively of thyroxine‡ subcutaneously. The thyroxine crystals were dissolved in distilled water with just enough 0.1 N NaOH added to make them soluble. The thyroxine solution was prepared weekly and stored in a refrigerator between injections. The body weight range of these animals was 150-180 g.

Results. The results of calculating the BMR's of normal rats and salivariectomized rats, 4-40 days postoperatively, are shown in

TABLE I. Comparison of BMR's of Salivariectomized and Intact Rats. Differences between experimental and normal groups are not statistically significant using the t-test.

		Mean BMR (Cal./M²/hr)		
		Days postoperative		
Rats	No.	4-6	18-20	38-40
Salivariectomized	8	44.4 ± 5.6	44.1 ± 6.2	40.1 ± 5.0
Normal	4	47.1 ± 6.9	46.6 ± 3.1	
"	4			41.2 ± 5.5

* Department of Physiology and Pharmacology paper No. 103.

† Thanks are due Lederle Laboratories for a generous supply of thiouracil.

‡ Crystalline thyroxine was supplied through the courtesy of G. S. Reed of the Squibb Institute for Medical Research. This material contained approximately 90% l-thyroxine and 10% d-thyroxine.

TABLE II. Data for Determination of Daily Thyroxine Secretion Rate in Rats. Differences in thyroid weights between respective normal and experimental groups are not statistically significant using the t-test.

Groups	Thiourea	Daily thyroxine dose, γ	No. animals	Mean initial body wt, g	Mean body wt at autopsy, g	Mean thyroid wt, mg %
Normal	No	0	7	161	183	6.08 ± .7
	Yes	0	6	166	185	15.4 ± 1.3
	"	3	6	159	175	10.7 ± 2.1
	"	6	6	164	182	7.2 ± .8
Experimental	No	0	5	156	180	6.14 ± .7
	Yes	0	6	153	169	16.6 ± 2.2
	"	3	6	153	172	9.78 ± 2.9
	"	6	6	151	167	5.9 ± 1.9

Table I. In spite of the fact that at the time of each determination the mean BMR of the control animals was higher than that of the experimental animals, this difference is not statistically significant when analysed with the t-test.

Experimental data used in determining the daily thyroxine secretion rate of salivariectomized and intact rats are shown in Table II. Differences between the corresponding normal and experimental groups receiving otherwise similar treatment are statistically insignificant when analyzed with the t-test. By plotting the thyroid gland weights from Table II against different groups of treated animals as shown in Fig. 1, an estimation of mean daily thyroxine secretion is obtained. For the control group of rats this value is approximately 6.6 γ thyroxine secreted per rat per day and for the experimental group, 5.7 γ/rat/day. Because l-thyroxine is the more active if not *the* active form of thyroxine(4), the true secretion rate of l-thyroxine would be 90% of these values or 5.9 γ and 5.1 γ/rat/day respectively. Converting these figures to a weight basis, the values become approximately 3.4 and 2.9 γ l-thyroxine secreted/100 g rat/day.

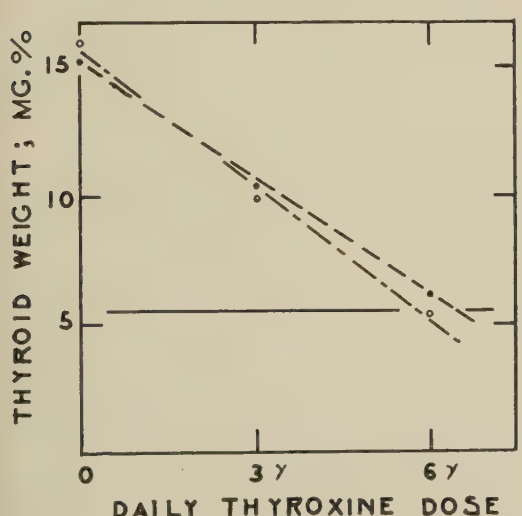

FIG. 1. Plotted data from which daily thyroxine secretion rate is calculated. ———, all rats receiving no thiouracil; – – – –, normal rats receiving thiouracil; — · — · —, salivariectomized rats receiving thiouracil.

Discussion. As a result of their experiments, Fawcett and Kirkwood(1) concluded that salivary glands of the rat are capable of acting as "thyroids-in-reverse" by degrading thyroxine into its constituent parts. This degradation by the salivary glands presumably occurs in steps, one of which(2) they have identified, namely, the deiodination of monoiodotyrosine under the control of tyrosine iodinase. This enzyme is reported to be comparatively abundant in the 3 salivary glands of the rat, particularly in the submaxillary and parotid glands. These 2 glands in the rat have greater concentrations of tyrosine iodinase than the thyroid gland where this enzyme catalyzes one step in thyroxine synthesis. In addition to the above action in the salivary glands, it has been reported that the normally rapid deiodination of intravenously injected diiodotyrosine does not occur in salivariectomized rats(1), at least within the space of 1 hour.

From the results of the present experiments it is concluded that the role of the rat's salivary glands in controlling thyroxine levels in

the body by fostering the degradation of thyroxine and/or its breakdown products is not an indispensable role under normal circumstances because the salivary glands can be removed without causing a statistically significant change in thyroxine metabolism as revealed by the BMR and thyroxine secretion rate.

Perhaps after salivariectomy, control of thyroxine level through degradation is assumed by other tissues at a slower but nonethe-less equally effective rate. The kidney and stomach have been reported to possess tyrosine iodinase in low concentrations(2). Evidence is accumulating to show that many tissues can deiodinate thyroxine with formation of triiodothyronine as for example the kidney in which the process appears to be controlled by an adaptive enzymatic mechanism(3).

On the other hand, an important point in determining the comprehensive role of the salivary glands in thyroxine metabolism still remains to be demonstrated; *i.e.,* their participation in the degradation of thyroxine itself to an extent relatively greater than that of other peripheral tissues which are metabolically stimulated by thyroxine and thus "utilize" thyroxine in their normal metabolism. If the salivary glands do not destroy thyroxine to a relatively greater extent than other tissues do, it is evident that a salivariectomy will probably not result in a recognizable change in BMR or thyroxine secretion rate.

Obviously more experimental data need to be available before an adequate appraisal of the normal role of the salivary glands in thyroxine metabolism can be made, however, in view of the present finding, it appears that this role is nonessential or adaptive.

Summary. Salivariectomy performed in young adult female rats produced no statistically significant effect on BMR or thyroxine secretion rate. It is concluded that rat salivary glands play a nonessential or adaptive role in thyroxine production and metabolism.

NOTE: After this paper was submitted for publication, two other papers(5,6) reported similar conclusions reached by different methods.

The technical assistance of Miss Gwendolyn Slover is gratefully acknowledged.

1. Fawcett, D. M., and Kirkwood, S., *Science,* 1954, v120, 547.

2. Fawcett, D. M., *J. Biol. Chem.,* 1954, v209, 249.

3. Larson, F. C., Tomita, K., and Albright, E. C., *J. Clin. Endo. Metab.,* 1955, v15, 836, (Abst.).

4. Reineke, E. P., and Turner, C. W., *Endo.,* 1945, v36, 200.

5. Ruegamer, W. R., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 146.

6. Tong, W., Potter, G. D., and Chaikoff, I. L., *Endo.,* 1955, v57, 636.

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Serological Response in Chickens to Beta-Propiolactone Treated Newcastle Disease Virus. (22240)

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In a previous study(1) it was demonstrated that Newcastle disease virus (NDV) was rendered noninfectious for chickens by treatment with 0.025% beta-propiolactone (BPL). The vaccinated birds developed no symptoms of Newcastle disease while 97.4% unvaccinated ones developed symptoms or died from the disease. Hemagglutination inhibition tests performed on paired sera from the vaccinated group showed a significant rise in titer.

The present communication presents additional information about the antibody response in the vaccinated group.

Materials and methods. White Leghorn chickens were used. All birds were from the same apparently disease-free flock. Beta-propiolactone was supplied by Dr. T. L. Gresham, B. F. Goodrich Research Center, Brecks-

TABLE I. Serological Response in Chickens to Newcastle Disease Virus Vaccine.

No. sera tested	Amt vaccine, ml	HI titer pre-vac,* avg	Post-vac, 16 day HI titer avg	No. sera tested	Pre-vac, serum index avg	Post-vac, serum index avg
13	.5	1:14	1:500	9	1.48	3.80
10	1.0	1:12	1:520	6	1.02	3.83
10	1.5	1:13	1:690	6	1.00	3.75
10	2.0	1:18	1:832	6	1.38	4.93
43	Avg	1:14	1:635	27	1.22	4.07

* vac = vaccination.

ville, Ohio. To facilitate dispensing of small volumes of BPL used, the BPL was diluted 1:10 with cold, sterile, distilled water and per cent concentration was based on undiluted BPL. In preliminary experiments, it was determined that treatment of NDV-infected allantoic fluid with 0.025% BPL for 2 hours at 37°C, destroyed infectivity of the virus for embryonated chicken eggs. It was also determined that heating NDV in allantoic fluid without added BPL for 2 hours at 37°C did not appreciably inactivate virus infectivity. Several serial embryonating egg passages were made with the virus by the allantoic route, prior to preparing a pooled allantoic fluid-virus suspension. The stock virus possessed a $10^{-9.5}$ LD_{50} titer when tested by injecting 0.1 ml of virus into embryonating eggs. All chickens in the experiment were bled from the heart to obtain pre-vaccination blood samples and stored in a frozen state. Thirty-nine birds were not vaccinated, and 43 birds were divided into 4 groups of 13, 10, 10 and 10 and received 0.5, 1.0, 1.5 and 2 ml vaccine respectively. On the 16th day following vaccination, the birds were again bled from the heart and the serum held in a frozen state until pre- and post-vaccination sera could be tested at the same time. The vaccinated and control birds were challenged by injecting 0.2 ml of virus intramuscularly. No evidence of infection occurred in the vaccinated group. Thirty-eight non-vaccinated controls died from the disease. *Standard hemagglutination inhibition* (HI) tests were performed on all paired sera. Incubation took place at room temperature and results of the tests were recorded at 15, 30 and 45 minutes. The hemagglutinating inhibition titers in 0.25 ml of undiluted serum were computed as the reciprocal of virus titer divided by the reciprocal of serum titer x dilution of the serum. *Serum neutralization* tests were performed in 8-10-day-old embryonating chicken eggs. Serial 10-fold dilutions of virus were prepared in nutrient broth. Equal parts of undiluted serum to be tested were mixed with each virus dilution. After standing 30 minutes, 0.1 ml of each virus-serum mixture was injected into the allantoic sac of 5 eggs. Viral infectivity was determined by mortality of the embryos. The *serum neutralizing titers* were the difference between the logarithms of titers of virus incubated with and without antiserum.

Results. The serological responses in chickens receiving the vaccine are shown in Table I. The HI titer rose gradually in the serum samples as the amount of vaccine given was increased. The average pre-vaccination HI titer was 1:14 which increased to average 1:635 sixteen days after vaccination. This represents a 45-fold increase in HI antibody titer.

The serum neutralizing antibodies against NDV also showed an increase in the post-vaccination serum samples. The average serum neutralizing index before vaccination was 1.22 in the 27 sera tested. The average index, taken on the 16th day after vaccination, was 4.07 or nearly a 4-fold increase over the average pre-vaccination index.

Discussion. Beta-propiolactone appears to be an ideal viricide with which to destroy the infectivity of Newcastle disease virus. Twenty-five thousandths per cent BPL was sufficient to destroy the infectivity of the virus and still leaving it capable of stimulating antibody to a high level to prevent disease. Lo Grippo and Hartman(2) have shown that

the murine encephalomyelitis (MM virus), Eastern equine encephalomyelitis and rabies viruses were antigenic but no longer infectious to mice following treatment with 0.1% BPL. A greater concentration of BPL was required for inactivation of mouse brain suspensions of these viruses.

By using Newcastle disease virus and BPL the alteration of infectivity and antigenicity of the virus could be measured in the chicken, which is the natural host for the virus. The absence of infectivity with retention of high antigenicity indicated that BPL is ideal for manufacture of certain viral vaccines. This is especially true when viruses propagated in chicken eggs may become contaminated with other viral agents.

Summary. 1. A vaccine made by treating Newcastle disease virus wtih 0.025% beta-propiolactone was found not to be infectious to the chicken, its natural host. 2. The sera from chickens receiving the vaccine were found to contain protective antibodies 16 days following vaccination. 3. Paired sera collected before and after vaccination showed a marked rise in hemagglutination inhibition and neutralizing antibodies.

Journal Article 1844, Department of Microbiology and Public Health, Michigan Agri. Exp. Station

1. Mack, W. N., and Chotisen, A., *Poultry Sci.*, 1955, v34, 1010.

2. Lo Grippo, G. A., and Hartman, F. W., *J. Immunol.*, 1955, v75, 123.

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Growth of *Micrococcus lysodeikticus* as Substrate for Lysozyme.*† (22241)

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Need for a procedure whereby large quantities of cells of *Micrococcus lysodeikticus* can be prepared conveniently for use as substrate for lysozyme, was encountered in studies of lytic enzymes from mammalian tissues(1). Present methods for production of *M. lysodeikticus* depend mainly on surface culture on agar media(2-6). Although a number of reports describe growth on liquid media(7-14), they are concerned primarily with nutritional requirements of the organism or pertain to mass cultivation without regard to lysability.

The present paper describes a simple procedure for production of cells of *M. lysodeikticus* in submerged culture. The influence of certain nutrients on growth and lysability of the cells is reported.

Methods and materials. M. lysodeikticus, strain No. 19 of the collection of the Institute of Microbiology, Rutgers University, was used throughout. All inoculations were made with 24 to 36 hour old agar cultures suspended in sterile, distilled water. Growth studies were carried out in test tubes (19 x 150 mm) containing 10 ml of medium. The tubes were set in a rack mounted on a rotary shaker and incubated at 28°C with agitation. Growth measurements were made with a Coleman Junior Spectrophotometer at 635 mμ. When quantities of cells were required, 250 ml Erlenmeyer flasks containing 100 ml of media were inoculated and incubated at 28°C on a rotary shaker. Method of preparation of dried cells and assay of lysozyme activity have been described previously(1). Crystalline egg white lysozyme was purchased from Worthington Biochemical Corp. The peptones used were: Lactalysate, Trypticase, Gelysate,

* Paper of the Journal Series, N. J. Agri. Exp. Station, Rutgers University, State University of N. J., Departments of Agricultural Biochemistry and Agricultural Microbiology, New Brunswick.

† This work was presented in part before the Theobold Smith Society of the Soc. of Am. Bact. at Princeton University, Princeton, N. J., June 2, 1955.

Thiotone—Baltimore Biological Laboratories, Md.; N-Z Amine—Sheffield Farms, Inc., New York City; and Bacto-peptone, Bacto-tryptose, Neo-peptone, Proteose-peptone and Protone—Difco Laboratories, Detroit, Mich.

Results. M. lysodeikticus was cultivated on a medium containing yeast extract, 0.1%; glucose, 0.5%; NaCl 0.5% and Bacto-peptone, 0.5 or 1.0% at pH of 7.2 and on similar media with various components withdrawn singly to determine essentiality for growth. Development in the complete nutrient medium was satisfactory; however, by doubling the peptone content and omitting yeast extract and glucose, growth was even better. Omission of yeast extract from a medium with glucose resulted in definite inhibition of growth and may reflect a need for biotin(12). The most rapid development was obtained on a broth consisting of 1% peptone and 0.5% NaCl. Dried cell preparations from each medium were tested as substrates for lysozyme using 5, 10, 20 and 100 μg of crystalline enzyme in a 5.5 ml system at 25°C. Since linearity between enzyme concentration and activity begins to fall off above 20 μg of enzyme(1,15, 16), the activity on the linear part of the curve as well as non-linear portion may be compared. The most susceptible cells in respect to linear and extra-linear concentrations of enzyme were those from the medium which contained 1% Bacto-peptone and 0.5% NaCl. This medium also gave the best growth.

The results of tests when 10 different peptones were substituted singly for Bacto-peptone are shown by Fig. 1. No growth was obtained on Protone or Neo-peptone. Although there was greatest development with Bacto-tryptose, growth was preceded by a lag period of approximately 25 hours. Growth on Bacto-peptone showed the shortest lag. Cells from each of the 10 peptones were tested for susceptibility to lysozyme and rate of lysis at linear and non-linear concentrations of enzyme was greatest in cells which grew optimally. With cells from media supporting poor growth (curves 5 through 8), this relationship was not apparent. Susceptibility of cells from the medium containing Proteose-peptone was lower with high concentrations of the enzyme. This may reflect a decreased availability of sodium ion(16). Although rate and extent of growth on peptones 5 through 8 differed greatly, the cells were comparable with regard to lysability. Gelysate, for example, produced cells whose lysis was comparable to that of cells grown on Lactalysate which produced 2 times as much growth. Of the 10 peptones investigated, Bacto-tryptose‡ and Bacto-peptone‡ were chosen for further study.

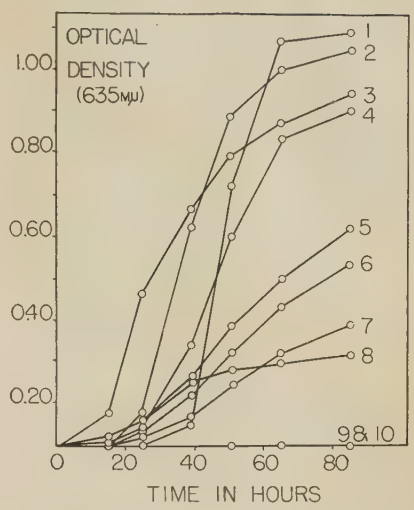

FIG. 1. Comparison of growth of *Micrococcus lysodeikticus* on 10 nitrogen sources. Curve 1—Bacto-tryptose, 2—Thiotone, 3—Bacto-peptone, 4—Proteose-peptone, 5—Lactalysate, 6—Trypticase, 7—N-Z Amine, 8—Gelysate, 9—Protone, 10—Neopeptone.

After demonstrating that the lag period for growth in Bacto-tryptose could not be eliminated by prior adaptation of the cells in this medium and that NaCl did affect the lag, cells were maintained on Bacto-tryptose agar and Bacto-peptone agar. These grew equally well when transferred to Bacto-peptone broth, however, addition of NaCl increased both extent and rate of development. Cells grown on Bacto-peptone agar and inoculated into Bacto-tryptose broth grew at a reduced rate compared to cells that had been maintained

‡ The composition of Bacto-peptone (Difco) and Bacto-tryptose (Difco) are presented in the Difco manual, Difco Laboratories, Inc. Detroit, Mich., 1953, pp. 256 and 262.

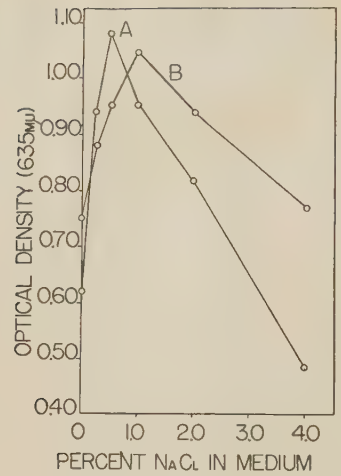

FIG. 2. Optimal concentration of NaCl for growth of *Micrococcus lysodeikticus* in Bacto-peptone and Bacto-tryptose media. Curve A, growth on Bacto-peptone; Curve B, growth on Bacto-tryptose.

on Bacto-tryptose agar. Cells maintained on Bacto-tryptose agar continued to show a prolonged lag period in Bacto-tryptose broth. This lag period was reduced markedly by addition of 0.5% NaCl to the medium. Cells that had been maintained on Bacto-peptone agar were influenced similarly by addition of NaCl.

The influence of concentrations of NaCl on growth of *M. lysodeikticus* in 64 hours is shown by Fig. 2. The optimal concentration of NaCl for growth was 0.5% with Bacto-peptone and 1% with Bacto-tryptose, even though Bacto-tryptose contains twice as much sodium ion and 10 times as much chloride ion as Bacto-peptone.‡

Tests of the activity of lysozyme on these cells indicated that susceptibility of cells grown on Bacto-peptone was greater than that of cells grown on Bacto-tryptose (Fig. 3). Bacto-peptone and Thiotone produced cells on which enzyme activity appeared to parallel growth. This was not the case for the other peptones.

Growth of *M. lysodeikticus* in Bacto-peptone broth was inhibited completely at pH 4 and 5. Although growth was most rapid at pH 7.5, it was comparable at pH 6 to 9 after 87 hours. Beers(14) also observed a high pH requirement for growth of this organism.

From the foregoing studies, the following medium is suggested for production of *M. lysodeikticus*: 1% Bacto-peptone and 0.5% NaCl in water at pH 7.5. Growth on this medium was compared with that on media recommended by Smolelis and Hartsell(6) and Saz and Krampitz(11). Agar was left out of the medium of Smolelis and Hartsell. All other components were equivalent. The inoculum was obtained from a Bacto-peptone-NaCl agar. The most rapid rate of growth was obtained on the recommended medium. There was considerable lag when *M. lysodeikticus* was grown in either of the other 2 media. The amount of growth was greatest on the medium of Smolelis and Hartsell. This is understandable since it contains as much as 6.5% available organic matter, as compared to 1.8% in the medium of Saz and Krampitz,

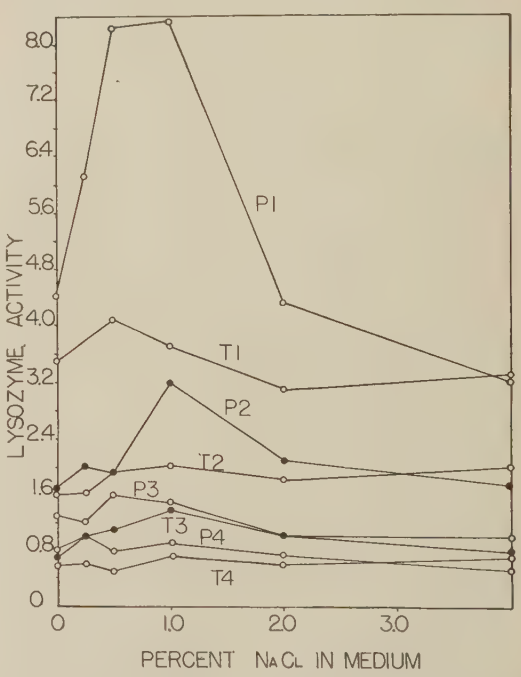

FIG. 3. Activity of crystalline lysozyme on cells grown on Bacto-peptone and Bacto-tryptose media containing various concentrations of NaCl.

Curve P1, cells from Bacto-peptone medium with 100 μg lysozyme.

T1	Bacto-tryptose	100
P2	Bacto-peptone	20
T2	Bacto-tryptose	20
P3	Bacto-peptone	10
T3	Bacto-tryptose	10
P4	Bacto-peptone	5
T4	Bacto-tryptose	5

TABLE I. Comparison of Lysozyme Activity on 3 Cell Preparations.

Medium used	Conc. of enzyme (μg)	Lysozyme activity* △ % transmission from 30-60 sec
S and H†	5	.5
	10	.7
	20	1.5
	100	3.5
S and K‡	5	.7
	10	.7
	20	1.7
	100	5.0
Peptone-salt§	5	.8
	10	1.0
	20	2.0
	100	7.9

* Duplicate determinations.
† Smolelis and Hartsell medium without agar(6).
‡ Saz and Krampitz(11).
§ The recommended peptone-salt medium.

and only 1% in the recommended medium. It is likely that the lag in growth on the medium of Smolelis and Hartsell could be partially eliminated by adjustment of pH. Likewise, there might be more rapid development in the medium of Saz and Krampitz by addition of NaCl and adjustment of pH.

Table I shows that the lysability of cells from the recommended medium is greater than that of cells from the other 2 media.

Since Wilcox and Daniel(16) have indicated that a decrease from linearity in enzyme activity at high concentrations of lysozyme may be partially overcome by addition of sodium ions to either buffer or enzyme solutions, the influence of NaCl added to suspensions of cells which had been grown in media containing from 0 to 4% NaCl, was tested. The results are shown in Fig. 4. Doubling and quadrupling concentrations of NaCl in the suspension of cells grown in Bacto-peptone containing 0 to 1% NaCl caused a progressive inhibition of enzyme activity, but did not influence the lysability of cells which were grown in broth containing 2 to 4% NaCl. If, at a concentration of 100 μg lysozyme/0.5 ml, sodium ion is required to increase enzyme activity(16) then increasing the concentration of NaCl in the suspension should influence lysability of cells grown in the absence of NaCl. This was not the

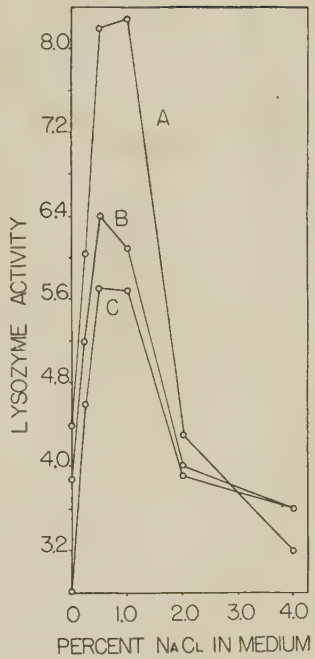

FIG. 4. Effect of adding NaCl to assay system on activity of crystalline lysozyme (100 μg). Cells grown in media containing varying amounts of NaCl. Curve A, .0171 M NaCl in assay system; Curve B, .0342 M; Curve C, .0684 M.

case. It is possible, therefore, that NaCl plays a structural role either in adsorption of the enzyme to the cell wall or in production of cellular substrate, or both; and consequently NaCl is more efficiently utilized if it is present during growth of the organisms.

Summary. (1) A broth consisting of 1% Bacto-peptone and 0.5% NaCl at pH 7.5 is recommended for growth of *M. lysodeikticus* as substrate for lysozyme. (2) Cells grown and prepared by the recommended procedure were more susceptible to lysozyme than cells produced by other methods. (3) The rate of lysis of cells of *M. lysodeikticus* was affected by the concentration of NaCl used in the culture medium.

The technical assistance of Mr. Harvey Oshrin in the preparation of cells and media is gratefully acknowledged. The authors are indebted to Professor R. L. Starkey of the Department of Agricultural Microbiology for reading the manuscript.

1. Litwack, G., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 401.

2. Boasson, E. H., *J. Immunol.*, 1938, v34, 281.
3. Epstein, L. A., and Chain, E., *Brit. J. Exp. Path.*, 1940, v21, 339.
4. Gale, E. F., and Epps, M. R., *Biochem. J.*, 1942, v36, 600.
5. Meyer, K., and Hahnel, E., *J. Biol. Chem.*, 1946, v163, 723.
6. Smolelis, A. N., and Hartsell, S. E., *J. Bact.*, 1949, v58, 731.
7. Gorini, L., *Biochim. et Biophys. Acta*, 1950, v6, 237.
8. Gorini, L., and Fromageot, C., *ibid.*, 1950, v5, 524.
9. Feiner, R. R., Meyer, K., and Steinberg, A., *J. Bact.*, 1946, v52, 375.
10. Salton, M. R. J., *J. Gen. Microbiol.*, 1953, v9, 512.
11. Saz, H. J., and Krampitz, L. O., *J. Bact.*, 1955, v69, 288.
12. Wessman, G. E., Allen, L. P., and Werkman, C. H., *ibid.*, 1954, v67, 554.
13. Wolin, H. L., and Naylor, H. B., *Bact. Proc.*, New York, N. Y., 1955, p47.
14. Beers, Jr., R. F., *Science*, 1955, v122, 1016.
15. Fraenkel-Conrat, H., *Arch. Biochem.*, 1950, v27, 109.
16. Wilcox, Jr., F. H., and Daniel, L. J., *Arch. Biochem. Biophys.*, 1954, v52, 305.

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Effect of Ergotamine on Milk-Ejection in Lactating Rat.* (22242)

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It has been generally accepted(1-5) that the oxytocic principle from the posterior pituitary gland is the humoral agent responsible for "let-down" of milk from mammary gland. It has further been demonstrated(6) that adrenalin, fright, and other stimuli, interfere with this effect, whereas acetylcholine, when injected, acts similarly to "let-down" hormone(7-8). Evidence is presented in this paper that ergotamine also interferes with normal "let-down" of milk in lactating rats.

Materials and methods. Docile lactating rats, each with its first litter and weighing 230-260 g, were housed in individual cages and given Purina Dog Chow and water *ad libitum*. Seasonal greens were fed twice weekly. Each litter was reduced to 6 young shortly after birth and, whenever possible, these were equally distributed as to sex. Rats with less than 6 young were discarded. Ten control and 10 experimental animals each with litter of 6 were used. Ergotamine tartrate‡ was injected subcutaneously into lactating mothers 3 times a day at 6-hour intervals, from ninth through twelfth days postpartum, at dosage of 3 mg/kg/day. Half of the controls received comparable injections of physiological saline to test whether or not frequency of injection and/or amount of fluid injected would affect the pattern of maternal behavior. The remaining controls were uninjected. One and one-half hours following the second of 3 injections of the day, each litter was weighed, then isolated from the mother for 6 hours during each day of injection period. The young were kept warm to prevent any undesirable effects from exposure. In some preliminary experiments, stomachs of several litters, killed at end of 6-hour isolation period, contained no milk. One and one-half hours after third injection of the day, each litter was again weighed to determine weight loss during the 6-hour interval. The young were then placed back with the mother and allowed to suckle for exactly 20 minutes. Length of time before each mother began nursing was recorded. The litter was then removed and weighed to determine amount of milk obtained during the 20-minute interval. (Table

* Part of dissertation submitted to Graduate School of Arts and Sciences of University of Cincinnati in partial fulfillment for degree of Doctor of Philosophy.

† Present address: Biology Department, University of Tennessee, Martin.

‡ Kindly supplied by Sandoz Chemicals, N. Y.

TABLE I. Average Quantity of Milk, Expressed as % Body Weight Gain, Secured in 20 Min. by Litters of 10 Control Lactating Rats, and 10 Experimental Animals Injected with Ergotamine from the 9th to 12th Days Postpartum.

Day of lactation	Group	%	S.E.	P
9	Control	5.59	.87	
	Treated	.36	.53	.001
10	Control	4.03	.54	
	Treated	2.22	.84	.1
11	Control	4.18	.69	
	Treated	2.46	.90	.2
12	Control	2.87	.82	
	Treated	2.27	.79	.7
Mean	Control	4.17	.73	
	Treated	1.83	.76	.001

I). All young were killed on twelfth day postpartum immediately after they had suckled. Their stomachs were opened and semisolid curds removed and weighed as a check against amount of milk calculated by weighing litters before and after sucking.

Results. Control offspring lost an average of 2% (±.15%) of their body weight during isolation period of 6 hours on each of days 9 through 12 postpartum, whereas litters of experimental group lost 2.2% (±.14%) during the same period. This difference is significant (P = .02). There is, however, no apparent difference between the 2 groups in the time it takes for mothers to commence nursing after their offspring have been returned to them following the isolation period of 6 hours. All mothers were nursing their young within 5 minutes. The quantity of milk obtained by experimental litters on the first day of treatment was very small in comparison with that obtained by control offspring on the same day. The difference was highly significant (P = .001). This difference progressively diminished, however, following additional treatment on days 10, 11, and 12 as animals became more resistant to the drug. (P = .1, .2, and .7 respectively.) The average amount of milk obtained over the 4-day period of treatment by experimental rats, nevertheless, was significantly less (P = .001) than that obtained by control offspring over the same period.

The combined weights of the semisolid curds, removed from the stomachs of each litter after they had been killed averaged 20% less than values calculated from weighing litters before and after the 20-minute period of nursing (Table II). This indicated that the method used in determining quantity of milk secured by young was accurate.

Discussion. It was expected that young of ergotamine-treated rats would lose a significantly higher percentage of body weight than control animals during the 6-hour isolation period. Offspring of treated rats are significantly lighter in weight at end of period of treatment than are control litters. Being of smaller size, there is a relatively greater surface area for heat loss than in larger control offspring. Greater energy, therefore, is expended by the smaller animal to maintain integrity of its internal environment.

It is well known(9-12) that with interruption of suckling, the mammary gland becomes engorged with milk. It was observed in the present study that when young were isolated from the mother for 6 hours and then replaced, the mother hastily gathered her young for feeding, apparently to relieve the turgidity in her mammary glands.

It was observed that within 20 minutes after being so isolated, young of untreated animals stopped sucking. Offspring of ergotamine-treated rats, however, were still sucking actively at the end of this period and had to be pulled away from nipples. It was apparent that they had not secured all the milk they wanted. The results for untreated litters (Table I), though not obtained under identical conditions, are similar to those obtained by other investigators(13-14).

Our studies(15) have shown that ergotamine does not impair synthesis of milk in mammary gland nor does it affect behavior of

TABLE II. Comparison of Calculated Quantity of Milk Obtained on 12th Day Postpartum and That Obtained from Stomachs of Litters of 10 Control Lactating Rats, and 10 Experimental Animals. Data expressed as % body wt gain.

Group	Calculated	Obtained	% difference
Control	2.87	2.24	21.9
Treated	2.27	1.84	18.9

the mother as evidenced by incidence of sucking during period of treatment.

Ergotamine apparently interferes, in some way which can only be explained theoretically, with release of milk from the gland. It has been demonstrated that definite contractile myoepithelial cells are present in mammary glands of the rat which contract apparently in response to oxytocin released from the posterior pituitary gland by nerve impulses stimulated by nursing(16-18). These cells are abundantly distributed around the alveoli and ducts and are not innervated. They are, furthermore, ideally arranged for squeezing milk out of acini and from small ducts into large ducts.

It is therefore likely that ergotamine may exert its inhibitory influence on milk "let-down" in rat mammary gland by interfering in some way with normal action of oxytocin on myoepithelial cells. In view of our results, further experimentation is planned with hope that the mechanism of ergotamine inhibition of milk "let-down" will be clarified.

Summary. 1. The quantity of milk secured in 20 minutes by litters of ergotamine-treated rats on the first of 4 days treatment was small in comparison to that obtained by control offspring on the same day. The difference was significant. This difference progressively diminished, however, following additional treatment on the following 3 days, as the animals became more resistant to the drug. The average amount of milk obtained by experimental animals over the 4-day period of treatment, nevertheless, was significantly less than that obtained over the same period by control offspring. 2. The possible mechanism by which ergotamine interferes with "let-down" of milk from rat mammary gland is discussed.

1. Whittlestone, W. G., *Nature* (London), 1950, v166, 994.

2. ———, *J. Endo.,* 1952, v8, 89.

3. Whittlestone, W. G., Bassett, E. G., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 197.

4. Whittlestone, W. G., *ibid.,* 1952, v80, 191.

5. Ely, F., and Petersen, W. E., *J. Dairy Sci.,* 1941, v24, 24.

6. Petersen, W. E., and Ludwick, T. M., *Fed. Proc.,* 1942, v1, 66.

7. Whittlestone, W. G., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 194.

8. Petersen, W. E., *ibid.,* 1942, v50, 298.

9. Hooker, C. W., and Williams, W. L., *Endocrinology,* 1941, v28, 42.

10. Maeder, L. M. A., *Am. J. Anat.,* 1922, v31, 1.

11. Weatherford, H. L., *ibid.,* 1929, v44, 199.

12. Williams, W. L., *ibid.,* 1942, v71, 1.

13. Cox, W. M., and Mueller, A. J., *J. Nutrition,* 1937, v13, 249.

14. Mayer, O. T., *ibid.,* 1935, v10, 343.

15. Grosvenor, C. E., 1956.

16. Linzell, J. L., *J. Anat.,* 1952, v86, 49.

17. Swanson, E. W., and Turner, C. W., *J. Dairy Sci.,* 1941, v24, 635.

18. Richardson, K. C., *Proc. Roy. Soc.,* London, s. B., 1949, v136, 30.

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Regional Differences in Propagation of Spreading Cortical Depression in the Rabbit.* (22243)

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Leão(1) reported that in the rabbit spreading depression (SD) invaded the entire exposed surface of the stimulated hemisphere with the single exception of a small region medial to the parasagittal sulcus. This region appeared to be coextensive with area Rsg β of M. Rose(2), also with area 29d of Brodman(3) and area u of Droogleever-Fortuyn (4). Evidence has been presented by J. E. Rose and Woolsey(5) that this region is part of the rabbit's cingular area, the rest of which is situated on the medial aspect of the hemisphere.

In the present paper that part of the cingular area which is situated on the dorsal surface of the hemisphere will be indicated by "Cg." The dorso-lateral aspect routinely exposed, exclusive of Cg, will be called the "convexity" of the hemisphere. The invasion of Cg by SD was investigated, using as a criterion the slow potential change (SPC) which accompanies this phenomenon, in addition to the changes in the electrocorticogram (ECG) used by Leão. Marshall and his coworkers have shown, mostly in cats and monkeys in which SD is less easily obtained than in rabbits(6,7), that the production of this phenomenon can be facilitated in several ways (8-13). After having confirmed Leão's finding that SD does not normally propagate into Cg, it was attempted to make this area exhibit SD by application of the procedures described by Marshall *et al.*

Method. Rabbits were used exclusively. Cannulation of the trachea, jugular vein and femoral artery, and exposure of the cortex were performed under ether narcosis. During recording, the preparation was immobilized with Squibb's Intocostrin (about 5 units/kg bodyweight/hour) and maintained on artificial respiration. Blood pressure was recorded from the femoral artery when necessary. The electrodes were placed as shown in Fig. 1A. Electrode 1 was used for stimulation. Bipolar ECGs were led off from the convexity of the hemisphere and from Cg with electrode pairs 2-3 and 4-5, respectively. Slow potential changes with respect to an indifferent electrode contacting the pinna were recorded from electrodes 3 and 5. Recording and stimulating electrodes were of the silver-silver chloride type mounted on springs and resting lightly on the cortex. Electrocorticograms were recorded with an Offner electroencephalograph, slow potential changes were fed into d.c. amplifiers(14) and recorded on photographic paper with d'Arsonval-type galvanometers. Galvanic stimuli (3 to 5 volts), lasting for 2-3 seconds, were used.

Results. Spreading Depression in the Dorsal Part of the Cingular Area (Cg). The first stimulations elicit on the convexity of the

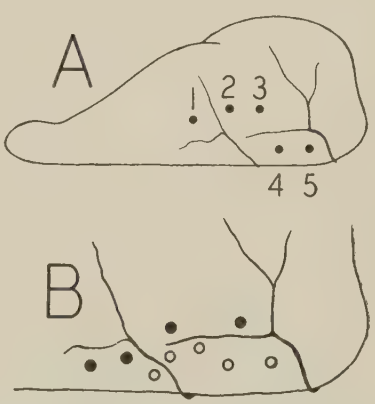

FIG. 1. A, a dorsal view of rabbit's hemisphere. Electrode placements are indicated by dots. Electrode 1 was used for stimulation. From electrodes 2 and 3 (on "convexity" of the hemisphere) and from electrodes 4 and 5 (on cingular area) bipolar electrocorticograms were led off. Slow potential changes were recorded from electrodes 3 and 5. B shows on a larger scale the cingular area on dorsal aspect of hemisphere. Electrode placements indicated by dots yielded typical slow potential changes, those indicated by open circles did not.

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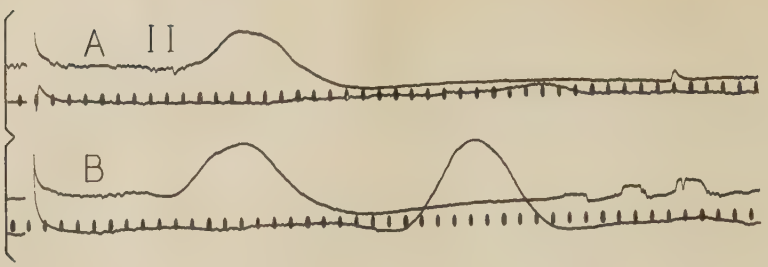

FIG. 2. Two slow potential change records, A and B. In each the upper trace is from convexity of hemispheres, lower trace from cingular area. A was made 33 min. and B 43 min. after start of infusion of 90% sucrose solution. Time marks indicate 10 sec intervals. The first 5 mV calibration line is for the upper, the second for the lower trace of each record.

hemisphere marked and often complete depressions of the spontaneous ECG accompanied by well developed slow potential changes (SPC). As the number of stimulations increases, depression tends to be replaced more and more by convulsoid patterns (1,6,15). The invasion of Cg by spreading depression (SD) was examined in 36 preparations. In 35 of them no typical SD or SPC invaded Cg. In most cases there was not the slightest indication of a slow potential change. In 9 preparations, however, a slight deflection was present in the SPC record from Cg. This small deflection appeared 1½ to 3 minutes later than the SPC recorded from the convexity of the hemisphere with electrode 3. Fig. 2A shows the relation in magnitude of the SPC recorded from the convexity and the small deflection recorded from Cg. A few spikes were sometimes observed in the ECG from Cg when the depression on the convexity of the hemisphere had been replaced by convulsoid activity. This spike activity, when present, appeared considerably later in Cg than on the convexity. Only in one preparation there was recorded from Cg a SPC comparable in size with that obtained from the convexity of the hemisphere. It was accompanied by a marked depression of the ECG. An attempt to delineate the region which does not produce the usual symptoms of SD is hampered by the long intervals between stimulations which are necessitated by the long refractory period of SD. Ten minute intervals were used in this investigation. Fig. 1B shows the results of an attempt to outline this region. It would appear that the limits of the unresponsive region correspond with that part of the area cingularis of J. E. Rose and Woolsey which is situated on the dorsal aspect of the hemisphere. In a few experiments the frontal boundary of the unresponsive area was traced as far as feasible on the medial aspect of the hemisphere. This boundary also appeared to coincide with that of Cg.

Effects of exposure of the cortex. In 7 preparations records made 2 to 3 hours after exposure of the cortex were not markedly different from the traces obtained shortly after removal of the dura. They showed clearcut SD and SPC in the traces recorded from the cortical convexity, no typical SD or SPC in Cg. Since long exposure of the cortex facilitates SD in monkeys(11) and cats(6,7) it was attempted to elicit this phenomenon in Cg of the rabbit in the same way. Two experiments were performed. In one, typical SDs and SPCs developed in Cg 8½ hours after exposure. They were present for two hours, after which the experiment was terminated. The other preparation examined for a similar period of time did not show a clearcut SPC in Cg.

Effects of intravenous administration of concentrated sugar solutions. Marshall(9) reported that dehydration of the cortex by slow infusion of a 90% sucrose solution facilitated the production of SD in cats. In the present experiments sucrose solution (90%) was infused through an inlying cannula into the jugular vein at a rate of 1-2 cc/min., causing a moderate blood pressure rise, profuse diuresis and in one preparation marked ascites. The brain shrank visibly. Three preparations were used, none of which exhib-

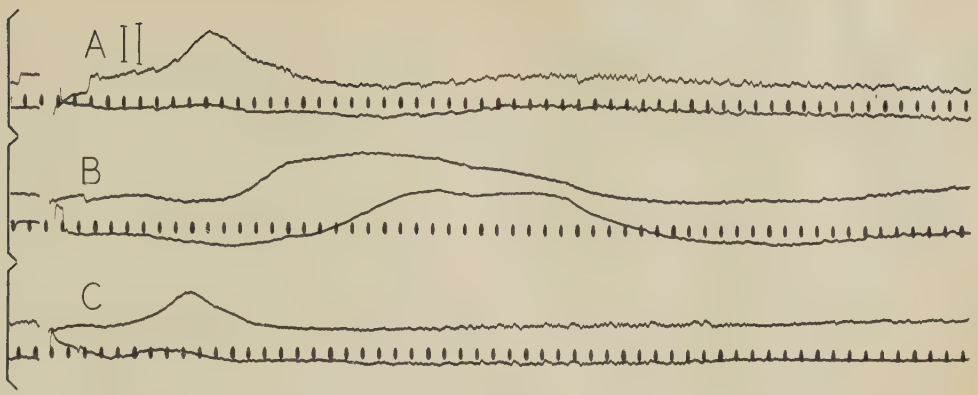

FIG. 3. Three slow potential change records, A, B and C. In each the upper trace is from convexity of hemisphere, the lower from cingular area. Record A was taken while cortex was covered with mineral oil near body temperature, B while cooled oil passed over cortex, and C again while covered by oil near body temperature. Time marks indicate 10 sec intervals. First 5 mV calibration line is for the upper, second for the lower trace of each record.

ited a typical SPC or SD in Cg before infusion. However, after 43, 50 and 53 min. of infusion a large SPC was recorded from Cg, accompanied by an incomplete but definite depression of the ECG. Fig. 2, A and B are SPC records produced by stimulations 33 and 43 minutes after the start of sugar infusion in one of these experiments. The upper trace of record A shows the well developed SPC led off from the convexity of the hemisphere. The lower trace recorded from Cg shows the small deflection mentioned above. Record B, taken 10 minutes later shows large SPCs of both the convexity and Cg, separated by a time interval of about 2 minutes. The duration of the depressions of the ECG recorded from the convexity of the hemisphere increased considerably during dehydration. Also, the height and duration of the SPC led off from the convexity were increased.

Effects of cooling of the cortex. Cooling the cortex was found by Marshall, Essig and Dubroff(10), to facilitate the production of SD. In the present experiments a skin well was formed over the exposed cortex by sewing the edges of the skin wound to a steel ring suspended a few cm. above the skull. The well was filled with oil near body temperature. Then cold oil (about 10°C) was passed through the well until a steady temperature of the outflowing oil was reached (17-20°C). Next the well was filled with warm oil again. After each change in oil temperature the cortex was stimulated. Fig. 3 is taken from such an experiment. Record A shows SPC traces from the convexity of the cortex (upper trace), and Cg (lower trace) respectively, while oil near body temperature filled the well. Record B was taken when the outflowing oil had reached a temperature of 18°C; and record C was made after a subsequent change to warm oil. Under oil near body temperature there is no obvious deflection in the SPC record from Cg, but after cooling there develops a large SPC of long duration which starts about 1 minute after the SPC recorded from the convexity. The SPC recorded from the convexity during cooling shows an increase in height and especially in duration (Fig. 3B, upper trace), as compared with the SPC led off from the same location under warm oil. Cooling increased the duration of the depression of the ECG from the convexity as much as 2 to 3 times, and had a tendency to decrease the convulsoid activity when present.

Effects of treating the cortex with isotonic sucrose solution. Essig and Marshall(8) reported that in cats and monkeys the production of SD is facilitated by treatment of the cortex with an isotonic sucrose solution. In the present experiments a solution of 10% sucrose in distilled water was made to flow at a rate of 10 to 12 cc/min. through the skin well described above. Since the solution has a high specific resistance, recording of ECG

and SPC can be continued during this procedure. Six experiments were successfully completed. Before application of the sugar solution typical spreading depressions were recorded from the convexity of the hemisphere but in none of the preparations from Cg. In all instances a sizeable SPC and definite depression of the electrocorticogram were recorded from this area after the cortex had been covered for some time (10 to 20 min.) by the sugar solution. The first stimulation after application of the sugar solution produced in each case a depression of the ECG of unusually long duration. Subsequent SD were of shorter duration and after 40 to 50 minutes of continued application of the sugar solution the depression was approximately of pre-treatment length. The SPC recorded from the convexity tended to be somewhat larger and of longer duration during the sugar application. In three experiments the sugar solution was replaced by Ringer's solution for about 10 minutes. The subsequent cortical stimulation caused a SD which was not propagated into Cg.

Effect of Ringer's Solution with 10 times normal potassium concentration. Treatment of the cortex with Ringer's solution containing more than the normal amount of potassium was also found to facilitate SD by Marshall *et al.*(12,13). In the experiments to be reported, KCl was added to normal Ringer's solution to make the K concentration 10 times normal. The solution was made to flow through the skin well at a rate of 10 to 12 cc/min. This treatment of the cortex caused spontaneous convulsive activity which, as was observed previously(16,17) can, and did act as stimulus for "spontaneous" SDs. By limiting the time of exposure in 2 experiments to 10 and 8 minutes, it was possible to avoid the convulsive activity so far that regular spreading depressions could be elicited. These preparations, which before the application of the K solution did not show a typical SPC or SD in Cg, did so after the cortex had been treated. After K treatment the magnitude of the SPC and the length of SD recorded from the convexity of the hemisphere were not increased. The cortex of the preparation with the 8 min. exposure was stimulated at 10 min. intervals following removal of the K solution. Only the first 2 stimuli produced typical SPCs in Cg; later stimuli although eliciting large SPCs on the convexity of the hemisphere failed to produce SPCs in Cg. The facilitation of the SD caused by exposure to the K solution thus seemed to be spontaneously reversible.

TABLE I. Latent Periods of Asphyxial Potentials from Convexity of Hemisphere and from Cingular Area (Cg).

No.	Convexity	Cg	Difference
1	310	460	150
2	125	230	105
3	130	270	140
4	140	280	140
5	120	160	40
6	80	85	5

Length of time, in sec between onset of asphyxia and development of the asphyxial potential. In preparations 1 to 5 the cingular area did not exhibit spreading depression; in preparation 6 it did.

Asphyxial potentials from the dorsal part of the cingular area. From 2 to 5 minutes after cortical asphyxiation a potential variation can be recorded from the cortex of similar magnitude and general appearance as the SPC accompanying the SD. Evidence has been advanced for the thesis that this asphyxial potential is related to the SPC accompanying SD(18-20). Since Cg is not normally invaded by the SD it was of interest to compare the asphyxial potential from the cortex on the convexity with the effects of asphyxiation of Cg. Six experiments were successfully completed. In 5 of them the SD did not invade Cg; the 6th experiment was the one, mentioned above, in which SPCs and depressions were recorded from this area. In all experiments an asphyxial potential was elicited both from the convexity and from Cg. However, in the 5 cases in which the SD had not invaded Cg the asphyxial potential developed from 40 to 150 sec. later in this area (Table I). In the experiment in which Cg exhibited SD the asphyxial potentials developed almost simultaneously.

Discussion. It is of interest that the limits of the area which is not normally invaded by SD correspond as far as investigated with the boundaries of the cingular area(5). There

thus seems to exist a relation between the presence or absence of SD and the anatomy of the cortex as given by cytoarchitectonic criteria.

On occasion a small deflection in the SPC record from Cg was observed. Since Cg is a strip of cortex only a few mm wide, electrodes placed on it are in general quite close to its lateral boundary in the parasagittal sulcus. Immediately lateral to the sulcus typical SDs and SPCs can be recorded. The distances involved are so small that the possibility has to be considered that the electrodes on Cg are affected by the physical spread of electrical phenomena occurring in the cortex immediately lateral to the sulcus. The time interval between the typical SD and SPC recorded from the hemispheral convexity and the atypical phenomena from Cg is commensurate with the time of propagation of the SD from the electrodes on the convexity to the cortex immediately lateral to the parasagittal sulcus. This explanation is consistent with experiments in which the propagation of SD was halted by a cortical cut(21); a small deflection similar to the one observed in the above experiments was recorded immediately across the cut on the non-stimulated side.

When after repeated stimulation the depression on the convexity of the hemisphere had been replaced by a convulsoid pattern, spike activity was sometimes observed in Cg. It seems possible that this spike activity was propagated actively from the cortex lateral of the parasagittal sulcus into Cg, since it is known that such convulsoid patterns are propagated to cortical areas not primarily affected(22). On the other hand, spread of a typical SPC into Cg is visualized as a change of this area, which makes it possible to be invaded by SD.

The 5 procedures which tend to facilitate the propagation of the SD in Cg produced changes in SD and SPC recorded from the convexity of the hemisphere which varied a great deal. Intravenous sucrose (90%) administration produced a gradual increase in the duration of the depression of cortical activity long before SD was propagated into Cg. Cooling caused a 100 to 200% lengthening of the depression. Treatment with isotonic sucrose solution produced an immediate prolongation of the depression, decreasing with the duration of the sugar application. The Ringer's solution with increased potassium concentration was without apparent effect on the length of the depression. Treatment of the cortex with isotonic sucrose solution, the intravenous administration of 90% sucrose, and especially cooling, increased and prolonged the SPC. The 5 procedures which facilitate the propagation of the SPC in Cg may not do so in the same manner. In the propagation of SD there may be involved a chain of events which can be facilitated at several points. For instance, it is conceivable that dehydration by bringing individual cortical elements closer together enhances the effect of one element on another. Or, if depolarization of neuronal elements is a necessary step in the propagation of SD(21) then this may be facilitated by the treatment with greater than normal potassium concentrations. If it be assumed that the 5 procedures facilitate via the same mechanism, then it follows from the varied effects of these procedures on the SPC of the convexity that this mechanism is not that underlying the changes in size, shape or duration of the SPC.

It was observed that the asphyxial potential develops later in Cg than in the cortex on the convexity of the hemisphere (in those preparations in which Cg is not invaded by SD). Assuming that the SPC accompanying the SD and the asphyxial potential are based on the same mechanism (18-20), then both this time difference and the fact that SD does not normally invade Cg may be indicative of the greater difficulty with which this mechanism is set in motion in Cg as compared with the cortex on the convexity.

Summary. 1. Of 36 rabbits consistently exhibiting spreading depressions and slow potential changes in response to galvanic stimulation of the cortex, only one exhibited propagation of these phenomena into that part of the area cingularis which is situated on the dorsal aspect of the hemisphere. Occasional small deflections recorded from this area were interpreted as evidence of physical

spread of typical slow potential changes in nearby cortex. 2. Where previously absent, typical slow potential changes and spreading depressions were produced in the cingular area by a) prolonged exposure of the cortex, b) intravenous infusion of concentrated sucrose solution, c) cooling of the cortex, d) treatment of the cortex with isotonic sucrose solution, and e) with Ringer's solution containing 10 times the normal amount of potassium. 3. Asphyxial potentials were recorded both from the cingular area and from the cortex on the convexity of the hemisphere. However, the latent period of this potential from the cingular area was markedly longer than that recorded from the convexity.

1. Leão, A. A. P., *J. Neurophysiol.,* 1944, v7, 359.
2. Rose, M., *J. Physiol. Neurol.,* Lpz., 1931, v43, 353.
3. Brodman, K., *Vergleichende Lokalisationslehre der Grosshirnrinde,* Leipzig, J. S. Barth, 1909, 324pp.
4. Droogleever-Fortuyn, A. B., *Arch. Neurol. Psychiat.,* Lond., 1914, v6, 221.
5. Rose, J. E., and Woolsey, C. N., *J. Comp. Neurol.,* 1948, v89, 279.
6. Sloan, N., and Jasper, H., *EEG Clin. Neurophysiol.,* 1950, v2, 59.
7. Van Harreveld, A., Stamm, J. S., and Christensen, E., *Am. J. Physiol.,* in press.
8. Essig, C. F., and Marshall, W. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 429.
9. Marshall, W. H., *EEG Clin. Neurophysiol.,* 1950, v2, 177.
10. Marshall, W. H., Essig, C. F., and Dubroff, S. J., *J. Neurophysiol.,* 1951, v14, 153.
11. Marshall, W. H., and Essig, C. F., *J. Neurophysiol.,* 1951, v14, 265.
12. Marshall, W. H., Essig, C. F., and Witkin, L. B., *Am. J. Physiol.,* 1951, v167, 808.
13. Marshall, W. H., Essig, C. F., Landefeld, M., and Witkin, L. B., *EEG Clin. Neurophysiol.,* 1952, v4, 244.
14. Van Harreveld, A., and Hawes, R. C., *Am. J. Physiol.,* 1946, v147, 669.
15. Whieldon, J. A., and Van Harreveld, A., *EEG Clin. Neurophysiol.,* 1950, v2, 49.
16. Van Harreveld, A., and Stamm, J. S., *J. Neurophysiol.,* 1954, v17, 505.
17. ———, *EEG Clin. Neurophysiol.,* 1955, v7, 363.
18. Leão, A. A. P., *J. Neurophysiol.,* 1947, v10, 409.
19. ———, *EEG Clin. Neurophysiol.,* 1951, v3, 315.
20. Van Harreveld, A., and Stamm, J. S., *Am. J. Physiol.,* 1953, v173, 171.
21. Van Harreveld, A., Terres, G., and Dernburg, E. A., *ibid.,* in press.
22. Van Harreveld, A., and Stamm, J. S., *J. Neurophysiol.,* 1953, v16, 352.

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Enzyme Studies in Muscular Dystrophy. II. Muscle Dipeptidase Activity and Vitamin E-Deficiency.* (22244)

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Vit. E-deficiency in the rabbit and other species results in characteristic muscular wasting and weakness. In previous investigations, on a number of tissues of the deficient rabbit, we found the cathepsin activity, *in vitro,* to be markedly increased in muscle, but unchanged in the other tissues(1). To investigate further the metabolism of protein in this form of muscular dystrophy, we have studied the activity of two previously characterized peptidase systems of muscle, glycyl-l-leucine and glycylglycine dipeptidase(2-4).

Methods. Vit. E-deficiency was induced in young rabbits by maintaining them on Diet 11 of Goettsch and Pappenheimer(5) treated with 1% ethereal $FeCl_3$. Control animals received the untreated diet supplemented with 25 mg % dl-α-tocopherol. After the appearance of symptoms of Stage II(6) of muscular

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TABLE I. Effect of Vit. E-Deficiency on Dipeptidase Activity of Muscle Extracts.

Substrate	Extract	Buffer	0.001M activator	Control: No. animals	Control: No. determ.	Control: Specific activity†	Vit. E-deficient: No. animals	Vit. E-deficient: No. determ.	Vit. E-deficient: Specific activity	t	P*
						60 min.			60 min.		
Glycyl-l-leucine	KCl	$PO_4^{\equiv}$	—	7	14	100 ± 38‡	6	10	410 ± 235	5.2	<.01
			$+Mn^{++}$	4	8	85 ± 29	5	8	300 ± 213	3.0	"
	H_2O	"	—	3	12	155 ±100	7	17	540 ± 250	5.3	"
			$+Mn^{++}$	3	6	75 ± 32	4	8	390 ± 237	5.3	"
						120 min.			120 min.		
Glycylglycine	KCl	"	—	5	10	65 ± 36	7	14	100 ± 59	1.8	<.1
			$+Co^{++}$	5	10	85 ± 32	7	14	410 ± 243	4.5	<.01
		Veronal	—	4	8	60 ± 10	7	14	80 ± 48	1.1	<.3
			$+Co^{++}$	4	8	80 ± 26	7	14	205 ± 99	3.8	<.01
	H_2O	$PO_4^{\equiv}$	—	4	8	110 ± 46	6	12	94 ± 37	1.0	<.4
			$+Co^{++}$	4	8	125 ± 53	6	12	405 ± 200	4.2	<.01
						mg protein N/ml			mg protein N/ml		
Protein nitrogen content of extracts	KCl			12		3.05 (2.19-3.72)§	12		2.45 (1.68-3.33)		
	H_2O			15		1.84 (1.41-2.52)	18		1.86 (.89-2.69)		

* P = Level of significance. † Specific activity = % hydrolysis/mg of muscle extract protein nitrogen/ml of incubation mixture. ‡ Stand. dev. § Figures in parentheses are range of values.

TABLE II. Effect of Vit. E-Deficiency on Dipeptidase Activity of Muscle Homogenates.*

Substrate	.001M activator	Control: No. of animals	Control: mg PN,† g wet wt	Control: Activity,‡ g wet wt	Control: Specific activity	Vit. E-deficient: No. of animals	Vit. E-deficient: mg PN, g wet wt	Vit. E-deficient: Activity, g wet wt	Vit. E-deficient: Specific activity
			a	b	b/a		a	b	b/a
Glycyl-l-leucine	—	4	23.5 (21.3–25.2)§	810 (710–1080)	34 (29–44)	3	19.2 (16.4–23.0)	5910 (2200–10800)	310 (95–660)
	Mn^{++}	3	24.2 (23.6–25.2)	700 (495–840)	29 (20–36)	3	19.2 (16.4–23.0)	3410 (1470–5950)	175 (65–365)
Glycylglycine	—	4	23.5 (21.3–25.2)	160 (0–480)	7 (0–20)	4	20.7 (16.4–25.0)	184 (90–280)	9 (5–16)
	$+Co^{++}$	4	23.5 (21.3–25.2)	350 (200–500)	15 (8–21)	4	20.7 (16.4–25.0)	2350 (500–4000)	110 (22–250)

* All muscle homogenates were prepared in H_2O. † PN = Protein nitrogen. ‡ Activity = % hydrolysis of glycyl-l-leucine at end of 60 min. incubation period and of glycylglycine at end of 120 min. of incubation. § Figures in parentheses are range of values.

dystrophy, the animals were sacrificed. Mixed portions of the muscles of the leg, thigh and back were homogenized with 2 volumes of either water or 2% KCl. The enzyme source was either a muscle extract obtained by centrifuging at 1400 x g, or the whole muscle homogenate. The extracts were diluted to 5-15 times their original volumes, and activity was determined at 2 or more different dilutions. The homogenates were diluted to 5-10 times their original volumes and strained through gauze. The observed values of activities of duplicate aliquots were averaged. Protein nitrogen content of all of the preparations was determined by a colorimetric nesslerization procedure. The assay system consisted of 0.15 ml buffer (1 M phosphate, pH 7.4-7.6 or 0.015 M veronal, pH 7.7-7.9), 0.15 ml 0.01 M metal ion activator when used, 0.5 ml 0.1 M substrate, 0.5 ml of the enzyme source, and sufficient water to make the final volume 1.5 ml. The mixtures were incubated at 37.5 $\pm$ .5°C for periods of one and 2 hours. Hydrolysis of the dipeptides was determined by the colorimetric ninhydrin procedure of Moore and Stein(7) as adapted by Schwartz and Engel(8). Preparations containing the enzyme source showed no measurable change in ninhydrin reacting material in the absence of substrate.

Results. Dipeptidase activities of the muscle extracts are shown in Table I and those of the whole homogenates are given in Table II. The incubation periods used were relatively short as compared to previous studies on these systems. Although the rates of hydrolysis of both substrates decreased somewhat during the second hour of incubation, as previously reported for rat muscle(9), the hydrolysis of glycylglycine had to be determined over a 2-hour period in order to obtain measurable values in the control group. Hydrolysis of glycyl-l-leucine is reported for one hour periods of incubation although in many experiments activity during the second hour also was determined. The addition of Mn^{++} did not increase glycyl-l-leucine hydrolysis by either the normal or E-deficient tissues, and, in fact, was somewhat inhibitory. We do not know why the Mn^{++} activation of glycyl-l-leucine dipeptidase activity reported by Smith(2,4) was not observed in these experiments; however, it is noteworthy that in at least one instance, this investigator also failed to note any stimulation by Mn^{++}(2). The addition of Co^{++} caused a slight increase in glycylglycine hydrolysis by the control group and a considerable increase by the E-deficient group. During vit. E-deficiency there was a marked increase in dipeptidase activity of the rabbit muscle extracts and whole homogenates. The increase in glycyl-l-leucine dipeptidase activity was not dependent upon the addition of Mn^{++}, but the increase in glycylglycine dipeptidase activity during vit. E-deficiency required the addition of Co^{++} activator. Elevation in dipeptidase activity was observed during both the first and the second hours of incubation, although the absolute rates of hydrolysis by both the control and deficient groups decreased slightly during the second hour. In one experiment, hydrolysis of glycyl-l-leucine by a mixture of extracts from control and vit. E-deficient rabbit muscle was equal to the sum of hydrolysis by the individual extracts. This result suggests that the difference between the activities of normal and E-deficient muscle was not due to the presence of either an inhibitor in the control group or of an activator in the deficient preparations. Although there was usually some decrease in protein nitrogen content of the muscle during the deficiency, which could give slightly higher values for the specific activity, this relatively small difference in nitrogen content cannot account for the large increases in dipeptidase activity we have observed.

An increased incorporation of glycine into muscle protein, *in vivo,* occurs, at least over short periods of time, in deficiency of vit. E (10). Therefore, the decrease in muscle mass that is observed in this condition appears to be due to an even greater increase of protein catabolism. Both dipeptidase and catheptic (1) activity of muscle is increased *in vitro* during the deficiency. It has been demonstrated that purified cathepsins have the ability, under proper conditions, to synthesize polypeptides *in vitro* by transpeptidization (11,12). While the functioning of these en-

zymes *in vivo* is yet to be demonstrated and other and more important enzyme systems are probably involved in protein metabolism, it is plausible to speculate that the net breakdown of muscle protein, observed *in vivo* during vit. E-deficiency, may be due to increase in rate, and a shift in the equilibrium of a reversible enzyme system(s) catalyzing peptide bond formation and breakdown.

Summary. The effect of vit. E-deficiency on hydrolysis of glycylglycine and glycyl-l-leucine by skeletal muscle extracts and homogenates was investigated. During this deficiency there was a marked increase in dipeptidase activity of both muscle extracts and homogenates. The increase in glycylglycine dipeptidase activity could be demonstrated only after the addition of Co^{++}.

1. Weinstock, I. M., Goldrich, A. D., and Milhorat, A. T., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 257.

2. Smith, E. L., *J. Biol. Chem.,* 1948, v173, 553.

3. ———, *ibid.,* 1948, v173, 571.

4. ———, *ibid.,* 1948, v176, 9.

5. Goettsch, M., and Pappenheimer, A. M., *J. Exp. Med.,* 1931, v54, 145.

6. Mackenzie, C. G., and McCollum, E. V., *J. Nutrition,* 1940, v19, 345.

7. Moore, S., and Stein, W. H., *J. Biol. Chem.,* 1948, v176, 367.

8. Schwartz, T. B., and Engel, F. L., *ibid.,* 1950, v184, 197.

9. Engel, F. L., and Schwartz, T. B., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 615.

10. Dinning, J. S., Sime, J. T., and Day, P. L., *J. Biol. Chem.,* 1955, v217, 205.

11. Jones, M. E., Hearn, W. R., Fried, M., and Fruton, J. S., *ibid.,* 1952, v195, 645.

12. Fruton, J. S., Hearn, W. R., Ingram, V. M., Wiggans, D. S., and Winitz, M., *ibid.,* 1953, v204, 891.

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Measurement of Cell Growth in Tissue Culture with a Phenol Reagent (Folin-Ciocalteau). (22245)

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In tissue culture studies, the determination of the amount of growth under varying environmental conditions is often of central importance. In this laboratory, the actual enumeration of the cells(1) proved a useful but laborious procedure(2-4); and studies were therefore undertaken to devise a rapid yet reliable method of measuring growth. Consideration was given to the direct turbidimetric measurement of the resuspended cells, the determination of total nitrogen by nesslerization, or of cell protein by precipitation procedures; but the method to be here described proved superior to any of these in its simplicity and reproducibility. Essentially, the technic is a modification of the colorimetric method of Lowry, *et al.*(5) for measuring protein, using a phenol reagent (Folin-Ciocalteau) for the development of color. As here described, the method is designed for use in cultures adherent to a glass surface, and overlaid with a fluid medium.

Materials. 1. Earle's salt solution(6) (or any similar balanced salt solution) for washing the cell layer in the culture flask. 2. Alkaline copper solution to dissolve the cells. This is kept as 2 stock solutions, A and B: A, Na_2CO_3, 200 g; NaOH, 40 g; NaK tartrate, 2 g; q.s. to 10 liters; B, $CuSO_4 \cdot 5H_2O$, 5 g; q.s. to 1 liter. (The tartrate was incorporated with the alkali, rather than the $CuSO_4$, because of the slow development of a precipitate in the copper-tartrate mixture.) Solutions A and B are mixed in the proportion of 50 parts of A to 1 part of B to form Lowry's solution C, which is prepared fresh daily.

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3. Folin-Ciocalteau reagent† (7). The reagent should be standardized by determining that concentration which gives the maximum and most stable color. This standardization need be carried out only once for each lot. Crystalline bovine albumin may be used as the reference standard. One part of a solution at 50 μg/ml is added to 5 parts of solution C. Six ml are then distributed into each of 6 cuvettes, and a parallel series is prepared using H_2O instead of the protein solution. Dilutions of the F-C reagent are prepared as indicated below:

Reagent	5	5	5	5	5	5
H_2O	4	5	6	7	8	9
Dilution	5/9	5/10	5/11	5/12	5/13	5/14

0.5 ml of each of these dilutions is rapidly blown into 1 cuvette† containing the protein solution, and 1 control cuvette containing H_2O. The color is read after 15, 30, 60, 90, 120, 180, and 240 minutes, in each case against the corresponding water control. That dilution of the F-C reagent which reaches maximum color within approximately ½ hour, and remains stable for 2 hours thereafter, is the one used in the test.

Details of procedure. Step 1. Washing of cultures. The culture flasks are drained of medium by inversion, and the adherent cell layer washed twice with Earle's salt solution (6). For the culture flasks previously described (2,8), with a surface area of approximately 15 sq cm, and a 15 ml capacity, an 8 ml wash is used. After the second wash the flasks are left inverted over clean gauze to drain for approximately 15 minutes, when clean stoppers are inserted. At this stage, the washed and dry cells may be stored in the original culture flasks for several days with no change in the protein analysis. *Step 2. Solution of cells in alkaline copper tartrate.* Ten ml of Lowry's reagent C(5) are added to the flasks with an automatic syringe and allowed to remain in contact with the cell layer for 10 minutes, after which the flasks are shaken manually to complete the dissolution of the cells. At this stage also, the flasks may be stored for several days with no loss in protein. *Step 3. Color development.* A biuret color is produced by the interaction of the alkaline copper sulfate tartrate solution C with the protein; and the intensity of this color may serve as a rough measure of the amount of cells, and thus of the amount of the solution which must be used in the final test in order to produce a color within the optimum measurable range (optical density = <0.4). When the color is not detectable, 2 ml is taken for analysis; when the color is pale violet, 0.5 ml will suffice; while with a deep violet color, the solution should be diluted with 1 or 2 volumes of solution C, and 0.5 ml taken for analysis. The appropriate amount of the cell solution is pipetted into a Coleman cuvette,† and solution C added to a total of 5 ml. One ml of H_2O is then added, and finally, 0.5 ml of the properly diluted F-C reagent is jetted in by a syringe. The rapid admixture of the color reagent with the solution is essential; and the slow addition of the reagent without such rapid admixture leads to erroneous results. The controls consist of 3 cuvettes, each containing 1 ml of crystalline bovine albumin at 50 μg/ml instead of the H_2O used in the test proper. A water blank is also run. Absorption is read after 30 minutes at 660 mμ; but the color remains stable for 2 hours.

Calculation of results. Results of the test are most simply expressed in terms of the bovine albumin equivalent of the unknown cell culture. For 4 different cell lines the ab-

TABLE I. Conversion of "Bovine Albumin Equivalent" of 8 Tissue Culture Strains to Cell Mass, Nitrogen and Number.

Cell line	Multiplying factors to convert bovine albumin equivalent (mg) to: Cell mass (mg)	Cell N (mg)	Cell count ($\times 10^6$)
Mouse fibroblast (Earle)	1.4	.18	.12
HeLa (Gey)	1.4	.16	.16
Intestinal epithelium (Henle)	1.5	.18	.16
Human leukemia (Osgood #111)	1.6	.17	.13
#39 "liver" (Chang)	1.6	.19	.18
KB (Eagle)	1.6	.18	.15
Human conjunctiva (Chang)	1.7	.18	.17
"Liver" (Henle)	1.8	.19	.14

† Available from the Fisher Scientific Co.

† Round 19 x 105 mm tube, 5.5 min ml capacity.

TABLE II. Progressive Growth of 3 Cell Strains as Measured by Protein Determinations and by Nuclei Counts.

Cell line			Days after planting culture 1	2	3	4
Human leukemia (Osgood #111)	Pd‡	$\mu g \pm$ S.D.*	784 ± 16	1217 ± 12	1694 ± 11	2520 ± 43
		V†	2%	1%	.64%	1.7%
	Cc‡	$\times 10^4$*	97 ± 6.6	144 ± 4.5	217 ± 9.3	391 ± 42
		V	6.8%	3.1%	4.3%	11%
	Ratio of protein, μg /Cc $\times 10^4$		8.1	8.5	7.8	6.4
HeLa (Gey)	Pd	$\mu g \pm$ S.D.	746 ± 19	1025 ± 53	1498 ± 60	1988 ± 159
		V	2.5%	5.2%	4%	8.0%
	Cc	$\times 10^4$	80 ± 5.8	101 ± 16	163 ± 15	267 ± 39
		V	7.2%	16%	9.2%	14%
	Ratio of protein, μg /Cc $\times 10^4$		9.3	10.1	9.2	7.4
Intestinal epithelium (Henle)	Pd	$\mu g \pm$ S.D.	454 ± 11	743 ± 39	1005 ± 34	1607 ± 176
		V	2.4%	5.3%	3.3%	11%
	Cc	$\times 10^4$	51 ± 4.2	77 ± 8.8	101 ± 12	204 ± 18
		V	8.1%	12%	12%	8.8%
	Ratio of protein, μg /Cc $\times 10^4$		8.9	9.6	10.0	7.9

* Mean of 4 replicate flasks $\pm$ stand. dev. (σ). † Pearson's coefficient of variation $\frac{\sigma \times 100}{\text{mean}}$. ‡ Pd = Protein determination; Cc = Cell count.

sorption curve was a straight line function of the amount of cells up to an optical density $\left(\log \frac{I_o}{I}\right)$ of 0.4.† For a given cell type, the bovine albumin equivalent as measured colorimetrically may be converted to cell count, mg dry weight, total nitrogen, protein nitrogen, or any other desired criterion, by appropriate determinations on test suspensions simultaneously measured by the method here described. Some of these conversion factors are indicated for 8 cell types in Table I.

An experiment to show the reliability of the protein determination as a measure of cell growth with 3 different cell lines is summarized in Table II. A number of replicate T-15 flasks were inoculated with each strain, and their protein content and cell count measured daily. The coefficient of variation in the protein content of 4 flasks varied from 1 to 11%, averaging 3%. The coefficient of variation in the cell count procedure varied from 3 to 16%, averaging 9.5%. With each cell line the ratio of the protein content to cell count remained essentially constant until the 4th day, when there was a smaller amount of protein per cell, presumably indicative of a smaller average cell size.

Summary. An analytical method for the measurement of cell growth in tissue culture is described, based on the Lowry method for the determination of protein, and employing a phenol reagent (Folin-Ciocalteau) for color development. The results, referred to a bovine serum albumin standard, may be converted to dry weight, nitrogen or cell count by appropriate conversion factors. Those factors are here given for 7 human cell lines and 1 mouse line.

1. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E., Jr., *J. Nat. Cancer Inst.*, 1951, v11, 773.

2. Eagle, H., *J. Biol. Chem.*, 1955, v214, 839.

3. ———, *J. Exp. Med.*, 1955, v102, 37.

4. ———, *ibid.*, 1955, v102, 595.

5. Lowry, O. H., Rosebrough, N. J., Fair, A. L., and Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.

6. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

7. Folin, O., and Ciocalteau, V., *J. Biol. Chem.*, 1927, v73, 627.

8. Earle, W. R., and Highouse, F., *J. Nat. Cancer Inst.*, 1954, v14, 841.

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Glycogen Stores in Glucagon-Treated Rats. I. Time Factors.* (22246)

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Glucagon is believed to cause hyperglycemia by promoting glycogenolysis in the liver. This is based on the following evidence which has been reviewed in detail recently (1) and which is largely indirect: 1) the hyperglycemic response to glucagon is decreased or absent in fasting, untreated diabetes, severe liver disease(2) and after evisceration (3) when the amount of liver glycogen is presumably low or absent; 2) glucagon is more effective when injected into the portal vein than by any other route of administration and is ineffective after occlusion of the liver circulation(2); 3) glucagon causes a sharp increase in splanchnic glucose production, as measured by venous catheterization in man (4); 4) destruction of the pancreatic alpha cells, presumably accompanied by the liberation of preformed glucagon, causes an immediate hyperglycemia with a decrease in liver glycogen(5); 5) glucagon promotes liver glycogenolysis *in vitro* by activating the phosphorylase system(6). Although these findings would lead one to expect a decrease of liver glycogen in intact animals treated with glucagon, this effect has not been clearly demonstrated. Bürger and Kramer(2) first reported a decrease in liver glycogen in dogs treated with glucagon. However, these pioneering results were obtained with crude preparations of glucagon heavily contaminated with insulin. Furthermore liver glycogen was determined in samples obtained by means of repeated biopsies, a traumatizing technic which may cause glycogenolysis in itself. Similarly, using an impure preparation of glucagon, Heard *et al.*(7) obtained a decrease in liver glycogen in normal rats, while Milman *et al.* (8) obtained similar results in vit. E-deficient rats. In contrast with these findings, the liver glycogen was found normal in rats chronically treated with small doses of glucagon(9), and elevated in animals treated with much larger doses(10).

The reasons for these unexpected results are not clear. It has been suggested(9) that since the glycogenolytic effect of small doses of glucagon is of short duration, the glucose liberated can be redeposited rapidly as liver glycogen, perhaps as a result of increased insulin secretion(11) or through a reversal of the phosphorylase reaction(10). Reports on the effect of glucagon on muscle glycogen are also contradictory and although glucagon does not stimulate muscle phosphorylase activity(6), it appears to inhibit the glycostatic effect of insulin in normal muscles(12,13) and to have a glycostatic effect of its own in the muscles of vit. E-deficient rats(8).

The purpose of this work was to investigate the effects of glucagon and of epinephrine *in vivo* on liver and muscle glycogen and on adrenal ascorbic acid. The latter was determined in order to investigate the possibility that glucagon may stimulate the pituitary-adrenal system as suggested by measurement of corticoid excretion(14) and by analogy with the effects of epinephrine(15).

Methods. Two hundred thirty-one adult Sprague-Dawley albino rats of both sexes were used.§ The animals, kept on a stock diet of Purina checkers, were allowed to eat *ad libitum* or were fasted for 24 hours, as indicated in the tables. Glucagon (Eli Lilly and Co., lot no. 208-158B-197‖; 0.5, 2.5 or 5.0 mg/kg) and epinephrine (0.15 mg/kg) were injected intraperitoneally. In addition, some of the fasted animals received simultaneous intraperitoneal injection of glucose (1 g/kg). After 2, 6, 12, or 24 hours, the animals were decapitated, both adrenal

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‖ Glucagon was a gift of Dr. O. K. Behrens of the Lilly Research Laboratories.

TABLE I. Effect of Glucagon on Liver and Muscle Glycogen and on Adrenal Ascorbic Acid (mg/100 g of Fresh Tissue).

No. of rats	Time after inj., hr	Fasting time, hr	Glucagon, mg/kg	Glucose, g/kg	Glycogen				Adrenal ascorbic acid	
					Liver		Muscle			
					Avg ± S.E.	% change	Avg ± S.E.	% change	Avg ± S.E.	% change
6	2		—		1884 ± 162		—		431 ± 24	
6	”		0.5		1697 ± 270	− 6	—		342 ± 32	− 21
6	”		2.5		624 ± 119	− 67*	—		361 ± 39	− 16
6	”		5		591 ± 114	− 69*	—		401 ± 15	− 7
9	”		—		4139 ± 541		467 ± 28		378 ± 17	
20	”		5		1349 ± 188	− 68*	530 ± 22	+13*	358 ± 9	− 6
6	”	24	—		—		—		379 ± 15	
6	”	”	0.1		—		—		312 ± 14	− 18
6	”	”	5		—		—		260 ± 34	− 31*
6	”	”	—		—		742 ± 63		—	
6	”	”	5		—		753 ± 47	+1.5	—	
5	”	”	—		579 ± 80		—		391 ± 11	
5	”	”	2.5		190 ± 35	− 67*	—		389 ± 16	− 1
6	”	”	—		459 ± 16		395 ± 30		359 ± 5	
6	”	”	5		30 ± 3	− 94*	408 ± 24	+3	262 ± 12	− 34*
6	”	”	—		438 ± 85		320 ± 25		346 ± 6	
6	”	”	5		47 ± 7	− 90*	409 ± 9	+27*	256 ± 13	− 26*
8	6	”	—	1	517 ± 47		487 ± 47		366 ± 19	
8	”	”	5	1	288 ± 55	− 56*	485 ± 12	− 0.4	320 ± 6	− 13
5	12	”	—	1	317 ± 34		—		374 ± 21	
5	”	”	2.5	1	164 ± 15	− 48*	—		376 ± 11	+ 1
8	”	”	—	1	1086 ± 26		496 ± 42		308 ± 26	
8	”	”	5	1	822 ± 14	− 25*	552 ± 29	+11	350 ± 11	+13
8	24	”	—	1	54 ± 9		419 ± 35		376 ± 29	
8	”	”	2.5	1	196 ± 33	+264*	387 ± 22	− 8	388 ± 10	+ 3
8	”	”	—	1	175 ± 31		349 ± 23		335 ± 15	
8	”	”	5	1	366 ± 25	+108*	487 ± 40	+39*	353 ± 12	+ 5
8	”	”	—	1	125 ± 34		290 ± 39			
8	”	”	5	1	405 ± 64	+224*	345 ± 6	+18		
6	”	”	—	1			909 ± 56			
6	”	”	5	1			879 ± 111	− 3.3		

* $P \leqq 0.05$.

TABLE II. Effect of Epinephrine on Liver and Muscle Glycogen and on Adrenal Ascorbic Acid (mg/100 g of Fresh Tissue).

No. of rats	Time after inj.	Fasting time, hr	Epinephrine, mg/kg	Glycogen — Liver — Avg ± S.E.	Liver — % change	Glycogen — Muscle — Avg ± S.E.	Muscle — % change	Adrenal ascorbic acid — Avg ± S.E.	Adrenal ascorbic acid — % change
6	20 min.	24	—	506 ± 27		377 ± 21		397 ± 37	
6	"	"	.15	144 ± 23	− 72*	267 ± 17	−29*	252 ± 18	−37*
6	2 hr	"	—	211 ± 12		301 ± 26		351 ± 25	
6	"	"	.15	538 ± 36	+153*	162 ± 27	−47*	233 ± 20	−49*
6	"	—	—	1082 ±121		339 ± 20		395 ± 20	
6	"	—	.15	1565 ±244	+ 44	247 ± 29	−28	301 ± 28	−24*

* $P \leq 0.05$.

glands and samples of liver and of gastrocnemius muscle were removed as rapidly as possible. Liver and muscle were dropped in tared centrifuge tubes containing 30% KOH and their glycogen content was determined according to the method of Good, Kramer, and Somogyi(16) supplemented by the method of Nelson(17). The adrenal ascorbic acid was determined according to the method of Roe and Kuether(18). Each group of rats treated with glucagon or epinephrine had its own control group of rats treated with saline. The statistical significance of the difference between the mean experimental and control values was calculated according to Fisher(19).

Results. The results are presented in Tables I and II. Table I indicates that: 1) 2 and 6 hours after the injection of glucagon there was a marked decrease in liver glycogen which was still noticeable 12 hours thereafter. This decrease appeared to have been greater in animals receiving larger doses of glucagon, and occurred in fed, as well as in fasted animals, whether receiving glucose or not. The lowest value reached was 28 mg/100 g of liver. Similar values of about 30 mg/100 g of fresh tissue were found in 12 animals. 2) Twenty-four hours after the injection of glucagon (2.5 or 5.0 mg/kg) there was a marked and significant increase in liver glycogen. 3) The injection of glucagon was followed by a small and inconstant increase in muscle glycogen. This was statistically significant only in three cases. 4) Two hours after the injection of the largest dose of glucagon (5.0 mg/kg), there was a decrease in adrenal ascorbic acid. This decrease occurred only in fasted animals in which liver glycogen reached very low levels and not in animals which had been fed or had received an injection of glucose. No decrease in adrenal ascorbic acid was found in animals killed 6, 12, or 24 hours after glucagon injection. Table II indicates that 20 minutes after the injection of epinephrine, liver glycogen, muscle glycogen and adrenal ascorbic acid were significantly reduced. Two hours after the injection, muscle glycogen and adrenal ascorbic acid were still below normal, while liver glycogen had increased to a value greater than that of the controls.

Discussion. The results demonstrate that the first effect of glucagon in all doses used is liver glycogenolysis. The absolute magnitude of this effect appears to be greater in animals receiving larger doses of glucagon and in fed animals having more liver glycogen, although the largest per cent decrease (about 90%) was found in animals the liver glycogen of which had been depleted by fasting. This decrease in liver glycogen tends to disappear after 12 hours and is followed 24 hours later by a marked increase. The reasons for this phenomenon are not clear. It has been suggested that, after a period of marked glycogenolysis, glucagon may cause an inversion of the phosphorylase reaction, however, glycostatic effect of glucagon never has been observed *in vivo*. In addition, much smaller doses of glucagon which cause hyperglycemia and therefore presumably stimulate the phosphorylase reaction *in vivo*, do not promote secondary glycogenesis(9). It may be suggested that the primary effect of glucagon, regardless of the dose, is an acceleration

of liver glycogenolysis and that the secondary increase in liver glycogen is the result of an increase in insulin secretion due to the accompanying hyperglycemia(20). In addition, the increase of liver glycogen may be favored by the release of ACTH and/or adrenal cortical hormone due to a stress-like effect of the primary glycogen depletion or of large nonphysiologic doses of glucagon. This hypothesis is supported by the following observations: 1) glucagon hyperglycemia stimulates insulin secretion(11), and 2) large doses of glucagon cause a marked decrease in adrenal ascorbic acid (Table I). The effect of the adrenals appears to be a permissive one only, since glucagon (2.5 mg/kg) may cause increase in liver glycogen without significant changes in adrenal ascorbic acid. In this respect, the action of glucagon would be similar to that of epinephrine which also causes: 1) hyperglycemia and, presumably, insulin secretion, 2) decrease in adrenal ascorbic acid, and 3) secondary increase of liver glycogen(15). In the case of epinephrine this compensatory response is very rapid and its effects are seen after 2 hours. In the case of glucagon the glycogenolytic effect disappears only after 12 to 24 hours, perhaps because large doses of glucagon are destroyed less rapidly than epinephrine, or perhaps because the effect of epinephrine on the adrenal cortex is more specific. In agreement with the observation that glucagon does not influence muscle phosphorylase, glucagon did not cause a decrease in muscle glycogen. The slight increase in muscle glycogen observed in these experiments and in those of others(8), may be not a direct and specific effect of glucagon, but a result of the secondary secretion of insulin. This, apparently, cannot overcome the glycogenolytic effect of epinephrine in muscle (Table II). Further experiments are in progress to investigate the role of adrenals, pancreas and pituitary in the glycostatic effects of glucagon.

Summary and conclusions. 1. Effects of epinephrine and various doses of glucagon on liver and muscle glycogen and on adrenal ascorbic acid were studied. 2. Twenty minutes after injection of epinephrine a decrease in liver and muscle glycogen, and in adrenal ascorbic acid were observed. Two hours after injection, liver glycogen was significantly higher than in controls, while muscle glycogen and adrenal ascorbic acid were still below normal. 3. Glucagon caused immediate decrease in liver glycogen. When large doses were used this decrease was followed by secondary increase to values greater than normal. 4. The glycostatic effect of large doses of glucagon in the liver was probably associated with an earlier decrease in adrenal ascorbic acid. 5. Glucagon caused an occasional slight increase of muscle glycogen. 6. It is suggested that the primary effect of epinephrine and of glucagon in all doses is an acceleration of liver glycogenolysis and that their glycostatic effects are due to secondary increases in secretion of insulin and/or adrenal cortical hormones.

1. Foà, P. P., *Adv. Int. Med.,* 1954, v6, 29.

2. Bürger, M., and Kramer, H., *Ztschr. ges. exper. Med.,* 1929, v65, 487 and v67, 441.

3. Ingle, D. J., Nezamis, J. E., and Humphrey, L. M., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 232.

4. Kibler, R. F., and Myers, J. D., *Am. J. Med.,* 1952, v13, 647, and 1953, v14, 753.

5. Weisberg, H. F., and Kaplan, C. M., *Fed. Proc.,* 1953, v12, 151.

6. Sutherland, E. W., and Cori, C. F., *J. Biol. Chem.,* 1951, v188, 531.

7. Heard, R. D. H., Lozinski, E., Stewart, L., and Stewart, R. D., *J. Biol. Chem.,* 1949, v172, 856.

8. Milman, A. E., Treacy, A. M., and Milhorat, A. T., *Fed. Proc.,* 1954, v13, 100.

9. Galansino, G., Weinstein, H. R., Magill, A. M., and Foà, P. P., *Amer. J. Physiol.,* 1955, v180, 27.

10. Root, M. A., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 108.

11. Foà, P. P., Santamaria, L., Weinstein, H. R, Berger, S., and Smith, J. A., *Amer. J. Physiol.,* 1952, v171, 32.

12. Snedecor, J. G., DeMeio, R. H., and Pincus, I. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 396.

13. Rodriguez-Candela, J. L., *Ciba Found. Colloquia, Endocrin.,* 1953, v4, 233.

14. Kirtley, W. R., Waife, S. O., Helmer, O. M., and Peck, F. B., *Diabetes,* 1953, v2, 345.

15. Long, C. N. H., *Ciba Found. Colloquia Endocrin.,* 1953, v6, 136.

16. Good, C. A., Kramer, H., and Somogyi, M.,

J. Biol. Chem., 1933, v100, 485.
17. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.
18. Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, v147, 399.
19. Fisher, R. A., *Statistical Methods for Research Workers.* Oliver and Boyd, Edinburgh, 1950.
20. Foà, P. P., Weinstein, H. R., and Smith, J. A., *Am. J. Physiol.*, 1949, v157, 197.

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Abnormalities of Urogenital System in Strain A x C Line 9935 Rats. (22247)

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At autopsy congenital absence of one kidney was observed in a rat of the strain A x C line 9935 colony maintained at the National Cancer Institute. Subsequently, a number of rats of this strain were observed to have one kidney missing, and a number were observed to have other urogenital abnormalities. Observations on the extent and incidence of these abnormal conditions are presented herein.

Materials and methods. The strain A x C line 9935 (black agouti irish) rats were obtained from Dr. W. F. Dunning* of the College of Medicine, Wayne University in October 1945. At that time the strain had been inbred by brother x sister matings for 30 generations. The strain A x C line 9935 was started in 1926 by Doctors Dunning and M. R. Curtis from a cross between the August line 1561 and the Copenhagen line 2331. Neither of these 2 strains has ever been observed to have any urogenital abnormality. Dr. Dunning indicated that an abnormality was first observed in March 1937 in animals of the 16th brother by sister generation of the subline (which is the one on which the present observations were made). The right testicle was absent in 5 of a group of 50 males in which methylcholanthrene was being injected. She has observed urogenital abnormalities in the hybrid progeny of a male of the 12th generation of this same subline outcrossed to an August line female. In 1946, she briefly described the urogenital anomaly as occurring with about equal frequency in the 2 sexes and varying in extent in them. Either the right or the left side might be involved. In affected females, an adrenal and an ovary were absent occasionally and in affected males, the testis was absent or atrophic more frequently(1). Following the initial observation in this laboratory a routine search for similar abnormalities was made in young animals (one to 18 days of age) and in adult animals (2 to 17 months of age) of inbred generations 30 through 34. The majority of the adult animals were breeding females and for them data on the size and number of litters produced were available for comparison in the normal and abnormal animals. When the strain A x C line 9935 rats, in which the abnormalities had been observed, were outcrossed to strain M-520, the abnormalities were observed in the F_1 progeny(2).

Results. The incidence of urogenital abnormalities in young and adult rats in shown in Table I. It will be observed that the incidence of urogenital abnormalities in older animals was slightly higher than that in younger animals. However, when the data were analyzed statistically this difference was not found to be significant in either the females or the males (for the females, $\chi^2 = 3.39$; $P = 0.07$, and for the males, $\chi^2 = 0.26$; P is between 0.5 and 0.7).

Animals with urogenital abnormalities always showed either the absence of one kidney or the presence of one cystic kidney. Each of the two manifestations was often associated with various other abnormal conditions. There was no significant difference in the frequency

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TABLE I. Incidence of Urogenital Abnormalities in Strain A × C Line 9935 Rats.

Age	Sex	Total	% abnormal	No. of animals with specific organs abnormal: Kidney	Ureter	Oviduct & horn of uterus	Ovary	Ovarian capsule	Testis	Epididymis & vas deferens	Vesicular gland
1-18 days	♀	748	17.9	134	110	99	5				
"	♂	813	16.9	137	95				20		
2-17 mo	♀	189	23.8	45	40	38		18			
"	♂	62	19.4	12	12				5	12	6

of the abnormalities of the kidneys between females and males ($\chi^2 = 1.3$; P is between 0.20 and 0.30). However, they occurred more often on the right side than on the left (182 right and 142 left).† The absence of a kidney was seen more frequently on the right side, (150 right and 67 left) and the cystic kidney was seen more frequently on the left (32 right and 75 left).

Among the 45 abnormal adult females there were 33 which lacked one kidney, the right one in 21 and the left in 12. There was no ureter in 26 cases, no uterine horn in 28, no ovarian capsule in 17, and no oviduct in 2. There was hypertrophy of the remaining kidney‡ in 10, an incomplete ureter in 3, and a cystic and incomplete uterine horn in 4. Twelve of the 45 females had a cystic kidney (3 right and 9 left). The ureter was cystic in 9 cases, the uterine horn incomplete in 3, the ovarian capsule cystic in one, and the opposite kidney hypertrophied in one. The ureter was missing in 2 cases and the uterine horn in one.

In the group of 134 abnormal young females, 91 lacked one kidney, the right one in 65 and the left in 26. The ureter was missing in 84 cases, the uterine horn in 81, and the ovary in 5. The remaining kidney and ureter were cystic in 2 cases, the remaining kidney was hypertrophied in one and cystic in one, and the uterine horn was incomplete in one. There was one animal in which both kidneys, ureters, and uterine horns were lacking. One kidney was cystic in 41 (11 right and 30 left) of the 134 females and both were cystic in one. The ureter was cystic in 25 and the uterine horn in one. The uterine horn was incomplete in 16 cases.

Among the 12 abnormal adult males, there were 8 which lacked one kidney (5 right and 3 left). The ureter was missing in 8, the epididymis in 5, the vas deferens in 6, and the vesicular gland in 4. Atrophy of the testis was observed in 5 cases (the testis of one of these was undescended), of the vesicular gland in 2, and of the epididymis in one. The opposite kidney was hypertrophied in 2 cases. One kidney of 4 (one right and 3 left) of the 12 abnormal males was cystic. The ureter was cystic in 3 cases and ended blindly in one.

Among the 137 abnormal young males, 85 lacked a kidney (59 right and 26 left) and one lacked both kidneys. The ureter was missing in 61 and the testis was undescended in 6. The remaining kidney was hypertrophied in 4 and cystic in 5, and the remaining ureter was cystic in 2. Fifty of the 137 abnormal young males had one cystic kidney (17 right and 33 left). The ureter was cystic in 33 and incomplete in one, and the testis was undescended in 14. There was one animal in which one kidney was atrophied and the testis undescended.

As previously mentioned, a large number of the adult animals used in this experiment were breeding females. Therefore, data on the sizes of the litters of normal and abnormal rats were available. A reduction in the average litter size of successive litters in animals, in which abnormalities were present, as compared with the average litter size of normal animals has been observed (Table II).

† These figures do not include 3 cases where either both kidneys were missing or were cystic, and 1 case where the kidney was atrophic.

‡ Kidneys of small groups of normal animals and of animals lacking one kidney were weighed on spiral spring balance of the Roller Smith Type. Weights (average weight of both kidneys in normal females was 0.87 g and in normal males was 1.1 g while that of one kidney in abnormal females was 1.4 g and in abnormal males was 2 g) confirmed the gross observation of hypertrophy of the remaining kidney.

TABLE II. Average Litter Size for Normal and Abnormal Strain A × C Line 9935 Female Rats.

Group	No. of animals	Avg litter size for successive litters 1	2	3	4	5	6	7	8	9	10
Normal	127	6.9	7.9	7.2	7.0	6.4	6.3	5.8	6.4	4.9	7.0
Abnormal	39	4.6	4.6	4.4	3.7	4.5	4.0	3.8	4.0	1.0	

Discussion. Urogenital abnormalities have been observed in animals by various workers. Hain and Robertson(3,4) observed anomalies, similar to those just described, in Wistar rats and in hybrids between the Wistar strain and a hooded variety. The anomalies were manifested in various ways similar to those described for the strain A x C line 9935 rats, but differed from them in that the abnormalities occurred only on the left side. In contrast to this observation, Faulconer(5) reported a case of hydronephrosis with accompanying abnormalities on the right side in a young female rat of the McCollum hooded strain.

Following small doses of X-rays in mice, Bagg(6-9) observed abnormalities of the viscera, principally of the kidney. These ranged from partial to more pronounced atrophy of one kidney, to absence of one or both kidneys. Hydronephrotic or polycystic kidneys were also observed. The abnormalities occurred as often on the right side as on the left and in females as often as in males. In the present study the findings agreed with this sex distribution, however, the abnormalities occurred more frequently on the right side than on the left.

Summary. The occurrence of congenital abnormalities of the urogenital system of strain A x C line 9935 rats has been described. The principal manifestations were the absence of one kidney or the presence of one cystic kidney. These were accompanied generally by other abnormal conditions. In females, they included a missing ureter, uterine horn, oviduct, ovarian capsule, or ovary; an incomplete ureter or uterine horn; a cystic ureter, uterine horn, or ovarian capsule; or hypertrophy of the remaining kidney, with variation in degree of abnormality. In males, they included a missing ureter, epididymis, vas deferens, or vesicular gland; atrophy of the testis, vesicular gland, or epididymis; cystic ureter; undescended testis; and hypertrophy of the remaining kidney, all also varying in degree.

1. Dunning, W. F., personal communications.
2. Morgan, W. C., *Anat. Rec.,* 1953, v115, 635.
3. Hain, A. M., and Robertson, E. M., *J. Anat.,* 1936, v70, 566.
4. ———, *ibid.,* 1937, v72, 83.
5. Faulconer, R. J., *Anat. Rec.,* 1949, v103, 69.
6. Bagg, H. J., PROC. SOC. EXP. BIOL. AND MED., 1923, v21, 146.
7. ———, *ibid.,* 1924, v21, 228.
8. ———, *ibid.,* 1925, v22, 271.
9. ———, *Am. J. Anat.,* 1925, v36, 275.

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Coenzyme A Changes in Liver, Spleen and Kidney of Rats with Infections of *Plasmodium berghei.* (22248)

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Previous studies(1) by one of us have shown that survival *in vitro* of the avian malarial parasite *Plasmodium lophurae* was prolonged when concentrates of coenzyme A (Co A) were added to culture media containing the extracellular organisms. Further work (2) has indicated that the parasites were able to accumulate the coenzyme but were unable to synthesize it. In addition, the concentration of Co A in infected chicken livers was approximately 40% lower than in controls, whereas in ducks the drop in concentration of Co A was not so marked. It appeared, therefore, that the depletion of Co A in the liver was correlated with the accumulation of this essential growth factor in the parasites, and in turn was related to the pathology of infection. The present study was undertaken to determine whether a similar situation existed in a mammalian host infected with a malarial parasite. For this purpose observations have been made on the changes in Co A content of livers, spleens, and kidneys of rats during the course of infection with *Plasmodium berghei.*

Materials and methods. The parasite. The Kisonga strain of *P. berghei* was used in this study. This parasite was obtained from Dr. I. H. Vincke and for the past 2 years has been passed biweekly in this laboratory in 40-60 g rats, from the colony of Dr. John B. Nelson, by the intraperitoneal inoculation of whole blood. *The host.* For experimental purposes Sprague-Dawley rats were used. Of 30 animals 25 days old and weighing an average of 48 g, 15 were infected and the remainder kept as controls. Another 30 animals, 116 days old, weighing an average of 204 g, were treated in a similar fashion. To initiate infection each animal was inoculated with 50,000 parasites intravenously. Thereafter, films were taken daily of tail venous blood in order to follow the parasitemia. Infected rats were fed Purina dog checkers and water *ad libitum.* The amount of food and water consumed by each rat was carefully measured every day and averaged for each of the 2 infected groups. The average amount of food and water consumed was then offered to corresponding control animals. Young infected animals usually refused to eat after the first week of infection while older rats appeared to have an unaffected appetite. Restricted dietary intake of control animals was undertaken in order to rule out any possible effects of starvation on tissue Co A content. *The assay.* At 4-day intervals 3 animals from each infected group and 3 from each control group were sacrificed by decapitation. Each rat was allowed to bleed out. An abdominal incision was made and the spleen, liver, and kidney were removed in that order. Immediately upon removal each organ was weighed, placed in a celluloid tube, and then quick-frozen by immersion in a mixture of alcohol and dry-ice. Tissues so frozen were stored in the deep freeze at –25°C until they could be treated for incorporation in the assay medium. Some tissues were stored in this manner for almost 2 months. To prepare for assay each was ground with 4 times its weight by volume of water in a glass homogenizer in an ice water bath. The homogenate was immediately placed in a bath of boiling water for 10 minutes and centrifuged. Co A was determined in the supernatant by microbiological assay, using the methods cited in the work with *P. lophurae*(2).

Results. Essential host-parasite data are described in Table I. In each of the 2 age groups parasitemia followed a course similar to that reported elsewhere(3). In addition changes in the ratio of organ to body weight from the 4th to the 12th days of infection are presented. In young rats the spleen to body weight ratio increased 850% over controls by the 12th day of infection, whereas in old animals the increase was about 600%. At the

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TABLE I. Host-Parasite Data.

Group	No.	Day of infection sacrificed	Age, days	Wt†	Parasites,† 10000 rbc	Ratio liver to body wt*	Ratio spleen to body wt*	Ratio kidney to body wt*
1 X	3	4	29	67	706	.053	.0095	.0062
2 X	3	8	33	94	3410	.067	.028	.0059
3 X	3	12	37	80	6520	.062	.036	.0059
4 X 13	1	16	41	96	<1	.052	.034	.0049
14	1	16		57	12600	.063	.022	.0086
1 Control	3		30	61		.041	.0038	.0053
2 "	3		34	86		.040	.0050	.0049
3 "	3		38	86		.037	.0031	.0046
4 "	2		42	86		.036	.0026	.0041
5 X	3	4	116	208	30	.032	.0043	.0035
6 X	3	8	120	208	550	.039	.012	.0034
7 X	3	12	124	212	720	.037	.017	.0035
8 X	3	16	128	218	1	.034	.013	.0036
9 X	3	20	132	223	<1	.035	.0090	.0037
5 Control	3		117	200		.028	.0027	.0037
6 "	3		121	203		.026	.0025	.0035
7 "	3		125	212		.029	.0021	.0035
8 "	3		129	211		.026	.0023	.0037
9 "	3		133	223		.031	.0024	.0036

* Avg. † Avg for surviving animals.

same time, the liver to body weight ratio in young animals was 63% greater, and one half that value in old animals. The kidney to body weight ratio showed an increase of 25% in the young animals and no change in old rats.

Co A content of liver, spleen, and kidney at various stages during the course of infection is described in Table II. In the livers of young rats, a drop in Co A concentration was evident by the 8th day of infection. The total Co A present remained about the same even though liver to body weight ratios were 50% greater than in controls. The average percentages dry weight for the livers of 4 infected animals and 4 control animals sacrificed on the 8th day of infection were respectively 25.6% and 28.7% indicating that the drop in concentration of Co A was not simply dilution due to higher water content. In the older animals neither the decrease in coenzyme concentration nor the increase in liver size was so marked. In the spleen, on the other hand, the concentration of Co A remained constant through the course of infection, while the total amount present was proportional to the size of the organ. The kidneys of young rats showed a slight drop in Co A concentration with no change in the total, while in the older animals no changes were apparent in either organ size or Co A content.

It should be mentioned here that no differences were noted in concentrations of free pantothenate found in the livers or spleens of experimental and control animals. In both young and old groups the range for liver was 4-10 μg per g tissue. In spleen the range for young animals was 2-3 μg per g and in the older groups 1-3 μg per g. In the kidneys, both infected and uninfected old rats showed a range of 19-26 μg/g, whereas infected young rats had concentrations of 6-11 μg/g as contrasted to 12-28 μg/g in uninfected young animals.

Discussion. Changes observed in liver Co A of rats infected with *P. berghei* were similar to those described for ducks with infections of *P. lophurae*. In both cases there was a marked drop in the concentration of Co A, but the concomitant enlargement of the liver resulted in a total Co A content approximating that of controls. However, in chickens infected with *P. lophurae*, both the concentration and total amount of Co A in the liver were reduced. It is of interest to note the results observed with rats 13 and 14 in group 4 X (Table II). Rat 14, sacrificed at the terminal stages of infection, showed a decrease in both concentration and total Co A,

TABLE II. Average Units of Coenzyme A

Group	Category*	Liver		Spleen		Kidney	
		Per g†	Per organ	Per g†	Per organ	Per g†	Per organ
1 X	Infected—Young	163	580	20	13	55	24
2 X		98	594	21	54	56	30
3 X		103	512	21	60	51	24
4 X 13		157	790	24	78	67	32
14		81	301	19	24	58	29
1 C	Restricted diet controls	192	486	20	5	67	22
2 C	—Young	174	596	18	8	68	29
3 C		162	522	18	5	72	28
4 C		172	509	19	5	53	19
5 X	Infected—Old	112	737	19	18	59	44
6 X		110	851	17	54	36	27
7 X		110	848	23	69	47	34
8 X		109	813	23	68	54	42
9 X		93	728	20	39	74	60
5 C	Restricted diet controls	129	713	15	8	54	40
6 C	—Old	135	704	14	7	49	35
7 C		151	913	21	9	50	37
8 C		126	701	17	9	60	46
9 C		112	760	18	9	47	38

* For more complete data on course of infection see Table I. † Fresh wt.

despite its much enlarged liver. Rat 13, killed at the same time, was well on its way to recovery. Its liver had a normal concentration of Co A, but was still considerably enlarged, so that the total Co A was above that seen in controls.

The short period of reduced food intake which accompanied the infection in young animals, and which was artificially produced in the corresponding uninfected group, evidently had little, if any, effect on the Co A concentration of the liver. Average Co A concentration in the 6 livers of rat groups sacrificed on the 8th and 12th day, at the height of infection, showed a large decrease in infected animals and values for controls intermediate to those reported in the literature. Livers of young infected rats contained 100 units of Co A per g and mature infected rats 110 units. Of the controls the young animals showed 168 units, whereas the older ones had 143 units per g liver. The disparity among values cited in the literature(4-6) and with these might be due to the different strains of rats used.

Kidneys of rats infected with *P. berghei* exhibited no appreciable changes in either the concentration or total Co A present. However, there was a marked difference in the concentration of free pantothenate in the kidneys of young infected and control animals. This may have resulted from increased reabsorption of free pantothenate in the infected group because of the heavy demands of the parasite on the host for Co A.

In contrast to the liver, Co A concentration in spleen remained constant whereas the total amount present was proportional to increase in size of the organ. This suggests that the cells involved in hyperplasia of the spleen, in response to the infection, have the same Co A content as those originally present, and is in accordance with the finding that spleens of chickens with infections of *Plasmodium gallinaceum* showed no changes in glucose metabolism(7).

In view of both present and past work, it seems that the ability to produce Co A may rank as one of those factors of innate immunity which determine the ultimate death or survival of the host. When the demands of the parasite are so great that the host cannot supply its own tissues with sufficient Co A, the animal succumbs, whereas the animal will survive if it can maintain the coenzyme supply until the forces of acquired immunity control the infection. In addition the decreased concentration of Co A in the livers of infected animals might be responsible for the decreased ability of that organ to convert

glucose to glycogen(8). In any case, the observed changes are in harmony with the hypothesis that in mammalian as well as in avian malaria, disturbance in Co A metabolism is significant in the pathogenic effects of the infection.

Summary. Co A concentration and total content in liver, spleen, and kidney of normal rats and rats with infections of *Plasmodium berghei* have been described. During the course of infection the concentration of Co A in the liver dropped but the total amount present was unchanged. In the spleen the concentration remained static, whereas the total was proportional to the increased size of the organ. The kidney showed no appreciable changes in Co A, but a decreased concentration of free pantothenate was apparent in young infected animals.

1. Trager, W., *J. Exp. Med.*, 1952, v96, 465.
2. ———, *J. Protozool.*, 1954, v1, 231.
3. Singer, I., Hadfield, R., and Lakonen, M., *J. Infect. Dis.*, 1955, v97, 15.
4. Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, 1948, v174, 37.
5. Klein, H. P., and Lipmann, F., *ibid.*, 1953, v203, 101.
6. Olson, R. E., and Kaplan, N. O., *ibid.*, 1948, v175, 515.
7. Moulder, J., and Taliaferro, W. H., *J. Infect. Dis.*, 1955, v97, 137.
8. Mercado, T. I., and von Brand, T., *Exp. Parasitol.*, 1954, v3, 259.

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Inhibition of Mumps and Influenza B Virus Multiplication by Synthetic Poly-D-Lysine.* (22249)

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It has been previously reported that the synthetic basic polypeptide, poly-L-lysine, protects the chick embryo for a period following inoculation with a number of animal viruses such as mumps virus(1), infectious bronchitis, Newcastle disease viruses(2), and influenza B virus(3). A comparative study using a synthetic basic polyelectrolyte, as polyvinylamine, showed that this polymer was not as effective a virus inhibitor as poly-L-lysine and was almost 5 times more toxic to the chick embryo(3). Proteolytic enzymes (4) in the chick embryo have been demonstrated to destroy poly-L-lysine injected into the allantoic fluid(5). Since this enzymatic destruction of the L-polypeptide might be a factor that limits its protective action against viruses and as D-polypeptides are usually less susceptible to enzymatic hydrolysis, we have studied the effectiveness of poly-D-lysine against mumps and influenza B viruses in the chick embryo.

Materials and methods. The mumps and influenza B Lee strain viruses† were cultivated in chick embryos according to a previously described procedure(1,3). The virus cultures and the procedures for inoculation, harvesting and estimation of the extent of virus multiplication by hemagglutination titrations, were similar to those used by Green and Stahmann(1,3). In all experiments fertile White Leghorn eggs were employed. Poly-D- and poly-L-lysine hydrochlorides of molecular weights of about 3,900 and 3,600 respectively (determined by end group analysis) were prepared by methods published elsewhere(6-8). The maximum non-lethal dose of poly-D-lysine was approximately 0.1 mg and that of poly-L-lysine was 1.0 mg per chick embryo.

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TABLE I. Reduction of Hemagglutination Titer of Mumps Virus in Allantoic Fluid of Chick Embryo Injected with Polylysine.

1st inj., 0.1 cc intra-allantoic	2nd inj., 0.1 cc intra-allantoic (1 hr later), E.I.D.†	Hemagglutination titer* — Individual allantoic fluids					Geom. mean	Log mean	Difference from control log
Saline	10	160	80	160	32	80	88	1.94	
.04 mg DPL‡	"	0	0	0	0	0	0		1.94
" LPL§	"	0	0	0	0	0	0		1.94
Saline	100	256	128	64	256	256	169	2.23	
.04 mg DPL	"	32	0	0	2	8	8	.83	1.40
" LPL	"	0	16	16	2	4	7	.90	1.33
Saline	1000	256	512	256	128	256	256	2.41	
.04 mg DPL	"	32	8	0	0	16	16	1.20	1.21
" LPL	"	64	0	4	8	0	13	1.40	1.31
Saline	10000	256	256	256	256	128	223	2.35	
.04 mg DPL	"	64	16	32	0	16	27	1.43	.92
" LPL	"	64	32	16	64	16	32	1.51	.84

* Expressed as the reciprocal. † E.I.D. = Embryo infectious dose. ‡ Poly-D-lysine of avg molecular wt 3900. § Poly-L-lysine of avg molecular wt 3600.

Results. (a) *Effect of poly-D- and poly-L-lysine on inhibition of mumps virus multiplication in chick embryo.* Data of Table I show comparative effectiveness of poly-D- and poly-L-lysine in their inhibition of mumps virus multiplication. Groups of 5 embryos were injected intraallantoically with 0.04 mg of each polypeptide followed one hour later with 10 to 10000 embryo infectious doses of mumps virus. Controls were injected with saline. After 6 days of incubation at 37°C the eggs were harvested and hemagglutination titers of the individual allantoic fluids determined. It has previously been shown that poly-L-lysine cannot be demonstrated in the allantoic fluid by hemagglutination technic longer than about 24 hours after injection into the allantoic sac of the chick embryo(5), however, poly-D-lysine was demonstrated by the same technic to persist in the embryo up to 36 hours after injection. The data of Table I show that poly-D-lysine is equally as effective in inhibiting mumps virus multiplication as poly-L-lysine. It was thought that a difference in the rate of destruction of poly-D-lysine and poly-L-lysine as it influences the antiviral activity might be shown at lower concentrations of the polypeptides. Table II summarizes the results obtained when 0.001 to 0.04 mg levels of poly-D- and poly-L-lysine were administered to embryos injected with 1000 embryo infectious doses of mumps virus. Although the effectiveness of polylysine had decreased considerably at the 0.001 mg level, the comparative effectiveness of both types of polypeptides remained essentially the same.

TABLE II. Effect of Different Levels of Polylysine on Hemagglutination Titer of Allantoic Fluids Infected with 1000 E.I.D. of Mumps Virus.

1st inj., 0.1 cc intra-allantoic	2nd inj., 0.1 cc intra-allantoic (1 hr later), E.I.D.	Hemagglutination titer of allantoic fluids*	Difference from control log
Saline	1000	256	
.04 mg DPL†	"	16	1.21
" LPL‡	"	13	1.31
Saline	"	256	
.08 mg DPL	"	0	2.41
" LPL	"	4	1.81
Saline	"	256	
.1 mg DPL§	"	0	2.41
" LPL	"	0	2.41
Saline	"	128	
.01 mg DPL	"	8	1.20
" LPL	"	11	1.05
Saline	"	256	
.001 mg DPL	"	38	.83
" LPL	"	32	.90

* Expressed as reciprocal of geometrical mean of hemagglutination titers of allantoic fluids from groups of 5 to 6 embryos obtained 6 days after inoculation.

† Poly-D-lysine of avg molecular wt 3900.

‡ Poly-L-lysine " " " " 3600.

§ Maximum of one survived of a number of experiments done when inj. with 0.1 mg of DPL plus various levels of virus, probably due to the toxicity level of DPL.

TABLE III. Reduction of Influenza B Multiplication in the Chick Embryo by Polylysine.

1st inj., 0.1 ml intra-allantoic	2nd inj., 0.1 ml intra-allantoic (1 hr later), $I.D._{50}$	Hemagglutination titer*	Diff. from control log
Saline	10	320	—
.04 mg DPL†	"	8	1.60
" LPL‡	"	5	1.81
Saline	100	640	—
.04 mg DPL	"	22	1.46
" LPL	"	19	1.53
Saline	10000	640	—
.04 mg DPL	"	78	.91
" LPL	"	80	.90

* Expressed as reciprocal of geometrical mean of hemagglutination titers of allantoic fluids from groups of embryos obtained 2 days after inoculation.

† Poly-D-lysine of avg molecular wt 3900.

‡ Poly-L-lysine " " " " 3600.

(b) *Comparative effect of poly-D- and poly-L-lysine on influenza B virus Lee strain.* The data of Table III show the comparison of poly-D- and poly-L-lysine on influenza B virus. The procedures followed were similar to those used with mumps virus with the exception that the incubation period was only 2 days. The results again show that the two polypeptides have approximately the same antiviral activity.

Discussion. These data demonstrate that poly-D-lysine inhibits mumps and influenza B virus multiplication equally as well as poly-L-lysine. The extent of the inhibition obtained by poly-L-lysine was in agreement with that reported by Green and Stahmann (1-3). Since data have been obtained indicating that poly-L-lysine is metabolized in the allantoic fluid(5), it was thought that the duration of the virus inhibitor activity may be limited by the rate of metabolism of the polypeptide. However, the parallel antiviral activity of both the L and D polypeptides would suggest that poly-D-lysine may also be metabolized or hydrolyzed by the enzymes of the allantoic fluid. This metabolism of the D-polypeptide was further indicated by experiments which demonstrated that poly-D-lysine could not be detected in allantoic fluid by its hemagglutination ability for periods greater than about 36 hours following injection of the D-polypeptide. These observations suggested that there was an enzyme whose specificity was such that it allowed hydrolysis of poly-D-lysine. Experiments were therefore undertaken to demonstrate more clearly the existence and action of this new poly-D-lysyl peptidase. In subsequent experiments of this laboratory, it was found that poly-D-lysine is in fact hydrolyzed by extracts of pancreas powder as rapidly as is poly-L-lysine(6).

Summary. Poly-D-lysine, though ten times more toxic, inhibited mumps and influenza B Lee strain viruses to approximately the same extent as poly-L-lysine in the allantoic sac of the chick embryo. As little as 0.001 mg per embryo of either lysine polypeptide was sufficient to inhibit high levels of viral infection. Evidence was obtained which suggested the existence in allantoic fluid of enzymes which hydrolyze both poly-D-lysine and poly-L-lysine.

1. Green, M., Stahmann, M. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 852.

2. Green, M., Stahmann, M. A., and Rasmussen, A. F., Jr., *ibid.*, 1953, v83, 641.

3. Green, M., and Stahmann, M. A., *ibid.*, 1954, v87, 507.

4. Moog, Florence, *Ann. N. Y. Acad. Sci.*, 1952, v55, 57.

5. Rubini, J. R., Stahmann, M. A., and Rasmussen, A. F., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 659.

6. Tsuyuki, Emee, Tsuyuki, H., and Stahmann, M. A., in press.

7. Becker, R. R., and Stahmann, M. A., *J. Am. Chem. Soc.*, 1952, v74, 38.

8. Ben-Ishai, D., and Berger, A., *J. Org. Chem.*, 1952, v17, 1564.

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Effect of Dietary Carbohydrate on Experimentally Induced Hypercholesteremia and Hyperbetalipoproteinemia in Rats.* (22250)

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(Introduced by F. J. Stare.)

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The biliary excretion of bile acids by rats is increased when corn starch is used as the dietary carbohydrate in lieu of sucrose(1). A major portion of 4-C^{14} cholesterol administered to the rat or to man is excreted as labeled bile acids in the bile(2,3), and feeding of high levels of cholesterol to rats results in an increased biliary excretion of bile acids(1). Feeding of bile acids with cholesterol to rats results in a marked hypercholesteremia and hyperbetalipoproteinemia(4). There is still some question(5,6), however, as to the principal mechanisms involved. Any factor influencing bile acid excretion might be reflecting variations in conversion of cholesterol to bile acids. The effects of feeding various carbohydrates on experimentally induced hypercholesteremia and hyperbetalipoproteinemia in the rat were, therefore, investigated.

Methods. Rats were male albinos weighing 250 g (range: 240-260 g) and were received from Charles River Laboratories. The animals were individually caged and given water and diet *ad libitum*. There was essentially equal caloric intake in experimental groups and mean weight gains were identical (about 2 g/day). The basal diet consisted of casein 20%, corn oil 8%, carbohydrate 56%, cholesterol‡ 5%, celluflour 5%, salts(7) 4%, vitamins and trace nutrients as previously described(8). In some diets 1.5% cholic acid§ was included. Diets will be designated in the text and Tables according to type of carbohydrate and presence of cholic acid. Rats were fed experimental diets for 28 days. Serum total cholesterol was determined by the method of Abell *et al.*(9); the serum beta-lipoproteins were determined by the method of Gofman *et al.*(10). Liver total cholesterol values were determined by modification of the method of Abell *et al.*(9).

Results. Effect of starch and sucrose feeding on cholate induced hypercholesteremia and hyperbetalipoproteinemia. The results of analyses for serum total cholesterol and beta-lipoprotein concentrations and liver total cholesterol concentrations are indicated in (Exp. 1) Table I. Rats of the starch-cholic groups had lower serum total cholesterol values (mean = 257 mg %) than the sucrose-cholic group (mean = 378 mg %) ($p < 0.01$). The liver total cholesterol concentration was strikingly elevated in both groups but to about the same extent. Approximately two-thirds of the beta-lipoproteins in all the animal patterns had migration rates between S_f 20 and S_f 60 with maximum concentration at approximately S_f 35 and negligible amounts below S_f 12.‖ The mean serum concentration of S_f 20-100 molecules of the starch-cholic group was 216 mg % compared to a mean value of 413 mg % for the sucrose-cholic group ($p < 0.01$). Probably these differences between the 2 dietary groups were related to the previously demonstrated(1) lesser excretion of bile acids by rats fed diets containing sucrose.

Effect of sucrose, fructose, and glucose feeding on cholate induced hypercholesteremia. Serum total cholesterol and serum beta-lipoprotein responses obtained after feeding cholesterol and cholic acid diets containing sucrose, fructose, and glucose for 28 days are

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‡ Cholesterol was furnished by Armour & Co.

§ Cholic acid was furnished by Mr. de Haen, Miles-Ames Research Div., Elkhart, Ind.

‖ Determination of quantity of lipoproteins was based on refractive indices established on pooled human sera.

TABLE I. Effect of Dietary Carbohydrate on Serum and Liver Cholesterol Concentrations and Serum Beta-Lipoprotein Concentrations in Rats.

Exp.	Diet*	No. of animals	Serum cholesterol, mg %	Liver cholesterol, mg %	Serum β-lipoproteins (mg %) S_f 0–11	12–20	20–100	100–400
1	Cholate-sucrose	19	378 ± 23 †	8560	20	67	413 ± 49†	128
	-starch	19	257 ± 12	8420	14	48	216 ± 13	8
2	Cholate-sucrose	10	414 ± 36		23	82	521	60
	-glucose	10	361 ± 29		22	95	417	20
	-fructose	10	411 ± 20		16	56	504	90
3	Sucrose	10	124 ± 4.5					
	Starch	10	106 ± 3.9					
4	Cholate-sucrose	10	380 ± 35					
	-sucrose sulfa‡	8	348 ± 28					
	-starch	8	270 ± 25					
	-starch sulfa	9	361 ± 23					

* Rats were fed for 28 days on diets described in text. Diets contained 5% cholesterol. Presence of cholic acid and type of carbohydrate are indicated in "Diet" column.

† All values are means ± stand. errors of the means.

‡ Sulfasuxidine was added at 0.2% of the diet where "sulfa" is indicated in the "Diet" column.

indicated in (Exp. 2) Table I. Serum cholesterol was elevated in each group. Rats fed glucose had somewhat lower serum cholesterol and beta-lipoprotein levels, although the difference was not of high order of statistical significance. The lower serum cholesterol levels in rats fed a glucose containing diet compared to those fed the sucrose diet corresponded with the previous finding that there was a slightly greater 24-hour biliary excretion of bile acids in the former group(1).

Serum cholesterol response when cholesterol was fed without cholic acid. Serum total cholesterol values obtained after feeding sucrose and starch containing diets with cholesterol but without cholic acid for 28 days are indicated in (Exp. 3) Table I. The sucrose group had a mean value of 124 mg % compared to 106 mg % for the starch group ($p < 0.01$). This finding probably indicates that the starch group has a lower serum cholesterol value due to its greater excretion of intrinsically derived bile acids(1).

Effect of adding sulfasuxidine to cholate containing diets. Variations in intestinal flora could explain the different effects of sucrose and starch on cholesterol-cholate induced hypercholesteremia. Therefore, 4 series of rats were fed the cholesterol and cholate containing diet. Two series were given starch as the dietary carbohydrate, and 2 were given sucrose. One of the starch groups and one of the sucrose groups were fed 0.2% of diet as sulfasuxidine. The cholate-sucrose, cholate-sucrose-sulfa, and cholate-starch-sulfa groups had similar mean serum cholesterol values, whereas the cholate-starch group had a lower ($p = 0.01$) mean serum cholesterol value (Exp. 4) Table I. It would thus seem that the gastrointestinal flora in animals fed a diet containing corn starch might contain some organisms which were influencing the metabolism of cholesterol or its catabolic end product, cholic acid. Norman(11) has shown that administration of chemotherapeutic agents to rats destroys gastrointestinal organisms responsible for hydrolysis of conjugated bile acids.

Thus, in cases where a greater biliary excretion of bile acids (as with feeding of corn starch) had been previously observed(1) a lesser degree of hypercholesteremia was observed when cholesterol or cholesterol and cholic acid were added to the diet. There is an inferred causal relationship between lower excretion of bile acids and increased cholesteremia, although the mechanisms involved and their relationship to dietary carbohydrate have not been established.

Summary. Male albino rats were fed diets

for 28 days in which the type of carbohydrate was varied and serum cholesterol and beta-lipoproteins were determined. 1. Rats fed diets containing sucrose as the carbohydrate and with added cholesterol and cholic acid had higher serum cholesterol and serum beta-lipoprotein concentrations than did control rats fed diets with corn starch substituted for sucrose. Liver cholesterol concentrations were not significantly different. 2. Feeding of sucrose, glucose, and fructose as the dietary carbohydrate had essentially equal effects on cholesterol-cholic acid induced hypercholesteremia. 3. Rats which were fed cholesterol containing diets without cholic acid and with corn starch as the carbohydrate had somewhat lower serum cholesterol values than did the controls which were fed sucrose. 4. When sulfasuxidine was added to sucrose containing diets, there was no change in serum cholesterol values; however, addition of sulfasuxidine to starch containing diets resulted in elevation of serum cholesterol values to the level of that in rats fed sucrose.

1. Portman, O. W., Mann, G. V., and Wysocki, A. P., *Arch. Biochem.*, 1955, v59, 224.

2. Siperstein, M. D., and Chaikoff, I. L., *Fed. Proc.*, 1955, v14, 760.

3. Siperstein, M. D., and Murray, A. W., *J. Clin. Invest.*, 1955, v34, 1449.

4. Page, I. H., and Brown, H. B., *Circulation*, 1952, v6, 681.

5. Byers, S. O., Biggs, M. W., and Gunning, B., *J. Exp. Med.*, 1953, v97, 511.

6. Frantz, I. D., and Hinkelman, B. T., *ibid.*, 1955, v101, 225.

7. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.

8. Portman, O. W., and Mann, G. V., *ibid.*, 1955, v213, 733.

9. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., *ibid.*, 1950, v187, 97.

10. Gofman, J. W., Lindgren, F. T., and Elliott, H., *ibid.*, 1949, v179, 973.

11. Norman, A., *Acta physiol. scand.*, 1955, v33, 99.

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Isolation of Partially Purified Human Plasmin (Fibrinolysin). (22251)

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Although many methods for the preparation of plasmin from blood have been described, the utilization of highly purified plasminogen as a starting material appeared to offer the possibility of providing much purer plasmin than any previously reported.

This paper describes the preparation of plasmin from purified plasminogen, and the alcoholic fractionation of the resulting solution to obtain a plasmin product which is stable at low temperature and, in terms of fibrinolytic activity, is far more potent than any previously described fibrinolysin.

Materials and methods. Plasminogen. Lyophilized powder prepared by the method developed in this laboratory(1) from human plasma fraction III†. *Streptokinase* (SK). Commercial Varidase (Lederle).‡ Lots Nos. 7-1089-165A and 7-1089-142A were used. These contained 4,700 and 3,000 SK units per mg, respectively. *Fibrinogen.* Bovine Fraction I (Armour) was reprecipitated three times by the method of Laki(2) and was lyophilized. The critical assays were rechecked with fibrinogen which was reprecipitated seven times to eliminate the possibility that contaminating bo-

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vine plasminogen might have contributed to the results obtained(3,4). The quantity of fibrinogen in the lyophilized powder was calculated from nitrogen determinations, and a suitable amount was dissolved in phosphate buffer to a concentration of 0.4%. Fresh solutions were prepared for each series of assays. *Phosphate buffer.* 0.05 M, pH 7.8 for assays using 3 times purified fibrinogen; 0.01 M, pH 7.8 for 7 times reprecipitated fibrinogen. *Thrombin.* Commercial thrombin (Upjohn) was dissolved in an equal mixture of saline and glycerol to a concentration of 100 units per ml. *Assay of Fibrinolytic Activity.* A slight modification of the method of Ferguson(5) gave reproducible results. In one tube (8x70 mm), 0.5 ml of diluted enzyme solution, 0.5 ml of buffer and 0.04 ml of thrombin solution were mixed. To a second tube were added 0.5 ml of fibrinogen solution and 0.5 ml of buffer. The tubes were mixed immediately by pouring back and forth 3 times, placed in a water bath at 37.5°C, and the time of clot lysis determined. This tube contained a dilution of enzyme solution which had previously been found to dissolve the clot in the range of 270 to 330 seconds and was designated as 100%. Similar tubes containing 0.2 and 0.1 ml of diluted enzyme solution were made up to volume with buffer and were designated as 40 and 20%, respectively.

Units of Activity. The logarithm of the lysis time in seconds was plotted against the logarithm of the concentration (100, 40 or 20) and a straight line was drawn through the points. For a given fibrinogen solution, the slope of the line was constant. The activity of the 100% tube was arbitrarily assigned the value of 100 units. To obtain the activity per ml of original enzyme solution, the units obtained in each assay were multiplied by 2, since 0.5 ml is used in the assay, and then by the dilution factor. The specific activity is the activity per mg of nitrogen. *Activation.* The plasminogen powder was suspended in distilled water and dissolved by the addition of a few drops of N HCl. The concentration of enzyme was then adjusted to 4 mg/ml by the further addition of water. This solution was adjusted to pH 6.8 by the careful addition of dilute NaOH solution and 5,425 units of streptokinase per mg of plasminogen was added. The mixture was incubated at 37.5°C for 10 minutes after which the solution was adjusted to pH 8.7 and centrifuged. The pH was then lowered to 7.6 and again centrifuged, leaving a clear solution of plasmin. *Alcohol Fractionation.* The plasmin solution was chilled in an ice and salt bath to about 0°C and cold 95% ethyl alcohol was added dropwise with mechanical stirring until the desired alcohol concentration by volume was reached. The precipitate was permitted to settle overnight at 4°C, collected by centrifugation and dissolved in distilled water. A solution of plasmin so obtained has retained its original activity after seven months in the frozen state.

TABLE I. Alcohol Fractionation of 100 mg of Plasminogen after Streptokinase Activation.

% alc.	Nitrogen, mg/ml	Activity, units/ml (×1000)	Spec. activity, units/mg N (×1000)	Total activity in fraction,* units (×10⁶)
0	.67	40	60	1
10	.29	123	423	.6
21	.21	43.6	207	.2
Ppt. at pH 5.3	.89	4	4.5	.02
0	.85	40	47.06	1
10	.26	86	330.8	.4
0	.84	40	57.5	1
10	.46	70	151	.35
21	.32	21	66	.1
Ppt. at pH 5.3	.97	1.68	1.73	.01
0	.82	40	48.78	1
10	.37	100	294	.5
15	.21	35.6	170	.2
21	.14	17.8	127	.09
0	.76	40	53	1
21	.58	192	330	1
0	1.03	40	39	1
21	.76	155.2	203	.8

* Unfractionated solutions were 25 ml each. 0.5 ml of a 1:200 dilution was used in each assay. Total activity, therefore, was: 100 × 2 × 200 × 25 as described in the text. Alcohol fractions were dissolved in 5 ml of water which was then diluted 1:400 for assay.

Results. Table I shows the total and specific activities of the activated solutions, of the alcohol fractions, and of the remainder of the active material which precipitated at pH 5.3. It will be observed that the precipitate

obtained from 10% alcohol is the most active per mg of nitrogen, and that the fractionation is sharp, little activity remaining in the supernatant solution after removal of the 21% alcohol fraction. The combined activities recovered averaged about 75% of that present in the unfractionated solution, and the specific activity of the combined active fractions was about 3.5 times that of the starting solution.

To insure that these results did not represent the presence or absence of SK in any of the fractions, additional SK, 1000 units per assay, was added to each fraction with no effect upon the activity. In addition, to control SK to plasminogen ratios, 1000 units of SK per mg of enzyme nitrogen was added to each fraction assayed, again without affecting the activity appreciably.

Discussion. Although the plasmin obtained by streptokinase activation of purified plasminogen followed by alcoholic fractionation is the most potent fibrinolytic preparation thus far reported, the total yield and specific activity obtained may possibly be increased by varying the conditions of activation and fractionation. It is recognized that the amount of SK used in this procedure is quite large. In our laboratory, such amounts of SK alone or after mixing with plasminogen have regularly produced severe toxic effects and even death in the dog. Studies in dogs which are being carried out in the laboratory by Messrs. Fishbein and Newman, in collaboration with Dr. Louis Nahum, will be reported in detail elsewhere. Preliminary results indicate that the intravenous infusion of up to 1 mg/kg of purified plasmin is tolerated by the dog with no significant alterations of body temperature, blood pressure or electrocardiogram. Incoagulability of the dogs' blood appeared within 5 minutes of the injection. The following day, clotting times had returned to normal. The absence of toxic reactions in the dog of the new plasmin preparation would indicate that the toxic materials originally present in the SK(6) are largely eliminated by the fractionation procedure. Streptokinase itself is completely soluble in 21% alcohol. It is possible that a non-toxic SK-plasmin complex is the active form of the enzyme.

The dissolving of clots made from seven times reprecipitated fibrinogen would appear to rule out bovine plasminogen as an essential element of the clot-lysing system. The addition of 2 ml of human blood to 0.1 mg of purified plasmin resulted in immediate incoagulability. This experiment also eliminates the possibility that the release of heparin from the liver of the dog was responsible for the incoagulability observed. In preliminary experiments being carried out in association with Dr. W. Glenn the *in vivo* dissolving of a radiochromium labeled clot in the dog has been observed after the intravenous injection of 0.5 mg of plasmin per kg. The dissolving of the clot was confirmed by autopsy.

With the availability of this water-soluble, fibrinolytic enzyme preparation, preliminary clinical studies of the efficacy of this material in thrombotic conditions is now brought much closer. New light may also be thrown upon the physiology of blood coagulation by studies of the action of plasmin. Work is now in progress for the further purification and characterization of this protein.

Summary. A method for preparation and isolation of plasmin from purified human plasminogen after activation with streptokinase is presented. The product is water soluble, stable in the frozen state and produced no toxic manifestations in the dog after intravenous infusion of 1 mg/kg.

1. Kline, D. L., *J. Biol. Chem.*, 1953, v204, 949.

2. Laki, K., *Arch. Biochem. and Biophys.*, 1951, v32, 317.

3. Sherry, S., *J. Clin. Inv.*, 1954, v33, 1054.

4. Mullertz, S., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 291.

5. Lewis, J. H., and Ferguson, J., *Am. J. Physiol.*, 1952, v170, 636.

6. Tillett, W. S., Johnson, A. J., and McCarty, W. R., *J. Clin. Inv.*, 1955, v34, 169.

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Observations on Bound Ascorbic Acid in Guinea Pig Liver.* (22252)

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Sealock and co-workers(1,2) recently reported the presence of bound L-ascorbic acid in guinea pig liver. In their study, free L-ascorbic acid was extracted from liver with 95% ethanol and bound L-ascorbic acid was liberated from the alcohol-precipitated protein fraction by heating in acid solution. These investigators used the colorimetric method of Roe(3,4) to measure L-ascorbic acid present in the free and bound forms. The availability of L-ascorbic-1-C^{14} acid(5,6) has allowed further study of the presence of bound L-ascorbic acid in guinea pig liver. In addition, radioactive tracer technics have made possible observations on the binding of D-ascorbic acid in guinea pig liver.

Material and methods. L-ascorbic-1-C^{14} acid and D-ascorbic-1-C^{14} acid had specific activities of 1.40 and 1.18 μc per mg respectively. D-ascorbic-1-C^{14} acid was prepared by the method used for L-ascorbic-1-C^{14} acid (7-8) except that D-xylosone was used as the starting material instead of L-xylosone. Preparation of samples and assay for C^{14} were carried out as described previously(5). Male guinea pigs purchased from Rockland Farms, 2 to 3 months old and weighing 250 to 350 g, were used. Carrier dilution experiments(9) have shown that at least 95% of the C^{14} in the liver of guinea pigs 24 hours after intraperitoneal administration of L-ascorbic-1-C^{14} acid is present as L-ascorbic acid. It was therefore possible in the present study to determine the amounts of free and bound L-ascorbic acid by radioactive assay. Three normal guinea pigs and 2 guinea pigs maintained on a vit. C free diet for 14 days each received 3 mg of L-ascorbic-1-C^{14} acid by intraperitoneal injection. Twenty-four hours later the animals were sacrificed and their livers removed. A portion of the liver was homogenized with water and the total C^{14} in the liver was determined on an aliquot of the resulting homogenate by a wet combustion method(5). The remaining liver (5 g) was extracted 6 times with 30 ml portions of 95% ethanol by the procedure employed by Summerwell and Sealock(1). Measurements of C^{14} in the different alcoholic extracts showed that essentially all the free ascorbic acid was recovered in the first 3 extractions. The resulting alcohol-precipitated protein fraction was treated with hot 5% metaphosphoric acid(1) and after centrifugation a suitable aliquot of the supernate was assayed for C^{14} by the wet combustion technic(5). The % of ascorbic acid in the bound form was taken as the ratio of the total amount of C^{14} recovered after heat treatment of the alcohol-precipitated fraction to the total amount of C^{14} present in the liver. Further confirmation of C^{14} in the bound fraction being present as L-ascorbic acid was obtained by a carrier dilution technic(6). 200 mg of non-radioactive L-ascorbic acid was added prior to heat treatment and the C^{14} present in this fraction was recovered as the 2, 4 dinitrophenylosazone derivative of ascorbic acid. This technic has been shown to be highly specific for ascorbic acid. The determination of free and bound D-ascorbic acid in guinea pig liver was carried out in the same way as described for L-ascorbic acid. Two guinea pigs maintained on a vit. C-free diet for 14 days each received 3 mg of D-ascorbic-1-C^{14} acid by intraperitoneal injection and the animals were sacrificed 2 hr later and their livers removed. With D-ascorbic acid it was necessary to carry out the analyses at 2 instead of 24 hours used for L-ascorbic acid, because of the rapidity with which the former

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TABLE I. Bound L-Ascorbic Acid and D-Ascorbic Acid in Guinea Pig Liver, 7 Experiments.

Compound	Animal	% in bound fraction*
L-ascorbic-l-C^{14} acid	Normal guinea pig	14
Idem	*Idem*	23
"	"	20
"	Vit. C deficient guinea pig	31
"	*Idem*	25
D-ascorbic-l-C^{14} acid	"	35
Idem	"	34

* Expressed as % of C^{14} recovered after heating in acid of the alcohol-precipitated protein fraction to the total C^{14} present in the liver.

compound is eliminated by the guinea pig (10). A carrier dilution technic similar to that used for L-ascorbic-1-C^{14} acid showed that all the C^{14} in the liver 2 hours after administration of D-ascorbic-1-C^{14} acid is present as D-ascorbic acid(10).

Results. An average of 19% of the total L-ascorbic acid in the liver of the normal guinea pigs was found in the bound fraction compared to an average of 28% for the vit. C deficient animals (Table I). Sealock *et al.* (1,2) found that about 15% of the L-ascorbic acid in normal guinea pigs was in the bound fraction compared to 37% for vit. C deficient guinea pigs. Following the administration of D-ascorbic-1-C^{14} acid to vit. C deficient guinea pigs, about the same fraction of the total D-ascorbic acid in liver as in the case of L-ascorbic acid was found to be bound, averaging 35%.

Discussion. The results obtained in this study using C^{14} labeled L-ascorbic acid are in agreement with those determined colorimetrically by Sealock *et al.*(1,2) in that there is a bound form of L-ascorbic acid in guinea pig liver. At present no information is available on the nature of this binding. Sealock *et al.* (1,2) have attached importance to this bound form of L-ascorbic acid in the metabolism of tyrosine.

The results also show that D-ascorbic acid can exist in a bound form. In this connection it is of interest that D-ascorbic acid, which is reported to have no vit. C activity in guinea pigs(11), has recently been shown to be equally active as L-ascorbic acid in the *in vitro* metabolism of tyrosine(12).

Summary. Studies with C^{14} labeled L-ascorbic acid have yielded further evidence for the presence of a bound form of the vitamin in guinea pig liver. About 19% of the liver L-ascorbic acid is bound in normal and 28% in vit. C deficient animals. When D-ascorbic acid, the enantiomorph of L-ascorbic acid, is administered to vit. C-deficient guinea pigs, it was found in a bound form to about the same extent as L-ascorbic acid.

1. Summerwell, W. N., and Sealock, R. R., *J. Biol. Chem.*, 1952, v196, 753.

2. Sealock, R. R., Goodland, R. L., Summerwell, W. N., and Brierly, J. M., *ibid.*, 1952, v196, 761.

3. Roe, J. H., and Kuether, C. A., *ibid.*, 1943, v147, 399.

4. Roe, J. H., and Oesterling, M. J., *ibid.*, 1944, v152, 511.

5. Burns, J. J., Burch, H. B., and King, C. G., *ibid.*, 1951, v191, 501.

6. Burns, J. J., Mosbach, E. H., and Schulenberg, S., *ibid.*, 1954, v207, 679.

7. Burns, J. J., and King, C. G., *Science*, 1950, v111, 258.

8. Salomon, L. L., Burns, J. J., and King, C. G, *J. Am. Chem. Soc.*, 1952, v74, 5161.

9. Salomon, L. L., Doctoral thesis, Columbia University, New York, N. Y., 1952.

10. Burns, J. J., and Dayton, P. G., to be published.

11. Demole, V., *Biochem. J.*, 1934, v28, 770.

12. LaDu, B. N., Jr., and Greenberg, D. M., *Science*, 1953, v117, 111.

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Enhanced Absorption of Vitamin B_{12} in Gastrectomized Rat by Rat Intrinsic Factor.* (22253)

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The essential physiologic defect in Addisonian pernicious anemia and in the totally gastrectomized human subject is inability to assimilate vit. B_{12}. In both, this absorption defect can be diminished by the simultaneous administration of the so-called intrinsic factor of normal human gastric juice. This specific action of intrinsic factor is demonstrable following its simultaneous oral administration with vit. B_{12} in two ways: (a) hematopoietic effects in the anemic patient(1) or (b) enhanced uptake of radioactive vit. B_{12} in either the anemic or the non-anemic patient whether previously treated or not(2-4) including the gastrectomized patient(5). To date, no other method of detecting the activity of the intrinsic factor of normal human gastric juice has been found to be satisfactory.

The availability of radioactive vit. B_{12} labelled with Co^{60}(6), and especially of preparations of high specific radioactivity, suggested an attempt to develop an animal preparation for the detection of intrinsic factor. Although it was not known whether the rat, like man and the hog, secretes intrinsic factor, the availability of the species as a laboratory animal appeared to justify an effort to employ it. The fact that no increase, or indeed a decrease, in the uptake of radioactive vit. B_{12} had been found by others(7) to occur in the intact rat as a result of the simultaneous administration of intrinsic factor and vit. B_{12} was not discouraging, since this would be expected if the natural secretion of intrinsic factor was unimpaired and if non-specific binding(8) of Co^{60}-B_{12} by the intrinsic factor preparations had occurred. Our first interest was to discover whether the removal of the stomach and/or other portions of the gastrointestinal tract would reduce the ability of the rat to absorb radioactive vit. B_{12}. It was soon found, as recently reported by others(9), that with total gastrectomy this was so. However, it was considered that such a mutilating operation on the alimentary tract might well have had entirely non-specific effects on assimilation including vit. B_{12}. Consequently, only if an enhanced absorption of vit. B_{12} ensued in the operated animal when intrinsic factor was simultaneously administered by mouth could the conclusions be drawn with certainty that deficiency of intrinsic factor secretion (a) had been induced by the operative procedure and (b) had been temporarily abolished by the intrinsic factor administered. These conclusions appear to be justified by the present observations upon totally gastrectomized rats using Co^{60}-labelled radioactive vit. B_{12}, but only when normal rat stomach was the source of the intrinsic factor administered.

Methods. Male white rats of the Sprague-Dawley strain, weighing about 250 g were used. Total gastrectomy was done under Nembutal (pentobarbital sodium) anesthesia producing an end-to-end anastomosis of the lower end of the esophagus to the bisected pylorus. After brief experience the mortality at operation and during the following week was about 30%. Among animals surviving 2 weeks, only occasional animals lost weight and died. Gastrectomized rats were not used in experiments until at least 10 days after operation and only if they appeared to be in good health. At least 5 days elapsed between successive administrations of Co^{60}-B_{12} on the same gastrectomized animal. During the experiments the animals were kept in individual metabolism cages permitting total collection of stools without coprophagy. In order to

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minimize the bulk of the feces of which the radioactivity was to be measured, the diet supplied *ad libitum* consisted of a nutritionally complete synthetic low residue diet.‡ It contained 18% casein as the source of protein, and among other essentials 1.35 mg vit. B_{12}/100 lb diet. On this diet the gastrectomized rats appeared to be healthy, gained weight and excreted normal appearing feces. After a preliminary fast of at least an hour each animal was lightly anesthetized with ether and given a single dose of Co^{60}-B_{12} followed immediately by the preparation to be tested for intrinsic factor activity. Both were in liquid form and were injected with different syringes in measured amounts by means of a small rubber catheter about 3 mm in diameter that had been cautiously inserted through the esophagus for a distance of 8-10 cm. The amount of radioactive vit. B_{12}§ administered, which had a specific activity of about 1 $\mu c/\mu g$, was invariably 0.015 μg dissolved in 1 ml of water. The volume of the material to be tested for intrinsic factor activity was the same in each experimental group of rats and ranged in different experimental groups from 2-5 ml for a single dose. The stools were then collected over a 4-day period, digested with 20% NaOH and homogenized in a Waring Blendor. Thereafter 4 aliquot samples of 7.5 ml each were examined for radioactivity in a well-type scintillation counter and the individual values averaged. Human gastric juice was obtained from a normal fasting male following the injection of Histalog (3-beta aminoethylpyrazole dihydrochloride)(10). The juice was neutralized immediately after collection, passed through a coarse sintered glass filter and kept frozen until used. This procedure is known from many observations on patients with pernicious anemia(1) to provide an active source of intrinsic factor. As a source of hog intrinsic factor, a commercial preparation of hog stomach mucosa‖ was used. As a potential source of "rat intrinsic factor" the entire stomachs of normal rats were homogenized in a Waring Blendor with water. For each stomach, which had been kept frozen since excision and which weighed about 1.5 g, 10 ml of water were used in preparing the homogenate which was filtered through gauze in order to remove coarse particles before use.

Rat gastric juice was obtained from normal animals fasted overnight following ligation of the pylorus. In the morning a variable degree of gastric distention was noted when the animal was killed and the gastric contents, averaging about 15 ml, were removed. The gastric juice so obtained was slightly turbid and sometimes brownish in appearance. The pH of a pooled sample from several rats was 1.3.

Results. The results of measurements of the radioactivity in the feces in single experiments upon 11 normal rats and in 61 experiments upon 14 gastrectomized rats are graphically presented in Fig. 1. Values are reported as percentage recoveries of radioactivity after single oral doses of Co^{60}-B_{12} together with water or with one of the preparations to be tested for intrinsic factor activity.

In *normal rats* a significant fraction of the test dose of Co^{60}-B_{12} in water was usually absorbed as inferred from the excretion of radioactivity. In the *gastrectomized rats* there was apparently no absorption of Co^{60}-B_{12}. When 2 ml of normal human gastric juice were used as a source of intrinsic factor, the absorption of vit. B_{12} did not improve, nor was the fecal excretion diminished when, instead of 2 ml of normal human gastric juice given once, 3 ml were given initially and were followed after an interval of an hour by 3 ml more. The administration of 4 mg and even of 20 mg of the preparation of hog stomach mucosa was also ineffective in decreasing the excretion of radioactivity.

On the other hand, when whole rat stomach homogenized in water was administered in a dosage of ½ of a stomach to gastrectomized rats the absorption of vit. B_{12} apparently became normal. When the dose was decreased to 1/10 of a stomach, the effect upon fecal

‡ Available commercially from Nutritional Biochemicals Corp., Cleveland, O.

§ Kindly supplied by Dr. Nathaniel S. Ritter of Merck & Co., Rahway, N. J.

‖ Obtained through the courtesy of Dr. Kenneth W. Thompson of Organon Inc., Orange, N. J.

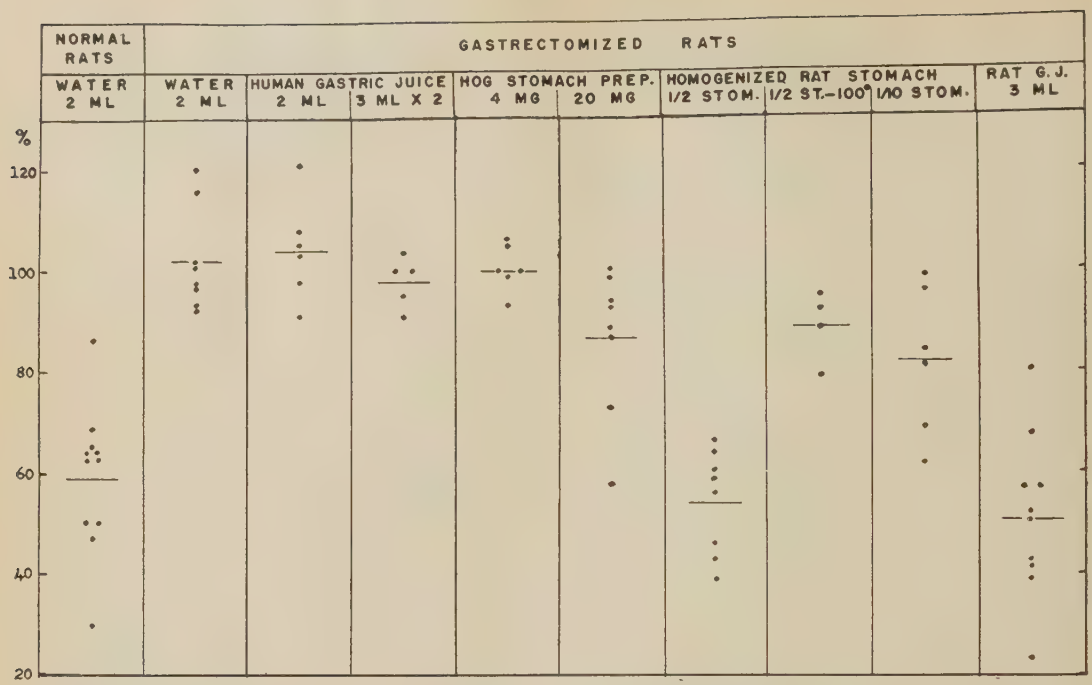

FIG. 1. Radioactivity recovered in the feces expressed as percentage of the single dose of 0.015 μg Co^{60}-B_{12} administered together with the substances indicated above. Each dot represents the result of an experiment on a single animal; bars indicate averages.

excretion was at most slight. When 3 ml of rat gastric juice were administered, the average reduction in fecal radioactivity resembled that obtained with ½ of a homogenized rat stomach. Finally, when ½ of a homogenized rat stomach was administered subsequent to heating on a boiling water bath for 7 minutes, its effectiveness appeared to be greatly diminished.

Discussion. These results appear to demonstrate that total gastrectomy in the rat renders the animal unable to absorb a small test dose of radioactive vitamin B_{12} when given in an amount of which about half is absorbed by the normal animal. This disability is not associated with any gross signs of intestinal dysfunction, and may be largely abolished by the simultaneous administration of sufficient homogenized rat stomach or rat gastric juice. Taken together, these findings indicate that the assimilation of vit. B_{12} in the rat resembles that in man in that the stomach appears to be the exclusive or major site of secretion of a so-called intrinsic factor essential for that process. However, neither normal human gastric juice nor the preparation of hog stomach mucosa employed displayed with certainty any activity as intrinsic factor in the rat.

During the preparation of this manuscript our attention was drawn to the recent publication of Watson and Florey(11) demonstrating that after resection merely of the distal (secretory) portion of the rat stomach results were obtained that were generally similar to ours. Thus, their operated animals failed to absorb radioactive vit. B_{12}, and this difference from the intact animal was corrected by administration of extracts prepared from the distal portion of the rat stomach but not by administration of the secretion from a pyloric pouch created in the hog. It is a pleasure to report this confirmation of their observations and to point to their additional evidence that in the rat the secretion of intrinsic factor is largely by the distal portion of the stomach.¶ Moreover, although not established by the experiments of Chow and his associates(9), it now appears probable that the defective absorption of Co^{60}-B_{12}

reported by them was in fact due to lack of rat intrinsic factor.

The apparent inactivity of normal human gastric juice and of hog stomach preparations employed by others(9,11) as well as here requires consideration. In our experiments both were obtained by methods repeatedly shown to conserve their intrinsic factor activity for patients with pernicious anemia. The doses of human gastric juice employed, 3 and 6 ml respectively, were for the rat relatively much larger than the 10 ml known to be hematopoietically active with as little as 1 μg of vit. B_{12} in pernicious anemia(12). The administration of 4 mg of the hog stomach preparation was without detectable effect as was also in all probability the administration of 20 mg. The larger amount constitutes about ½ of the amount of hog stomach extract present in 1 U.S.P. unit of a pharmaceutical preparation of vit. B_{12} with Intrinsic Factor Concentrate.** This is an effective daily oral dose in pernicious anemia when given with about 15 μg of vit. B_{12}. This hog stomach preparation contained about 80 $\mu\mu$g of normal vit. B_{12} in the 20 mg dose or far less than the 1680 $\mu\mu$g present in ½ of a homogenized rat stomach. Moreover, 6 ml of normal human gastric juice is said to contain only about 0.004 μg of vit. B_{12}(13). Thus, in these preparations insufficient normal vit. B_{12} was administered to compete significantly for absorption with the Co^{60}-B_{12} and so to account for their apparent inactivity in the rat. Although the inhibitory effect of non-specific binding(7,8) of the vit. B_{12} by the human and hog intrinsic factor preparations can not be excluded, the negative results with these preparations are consistent with the speculation of Chow and his associates (9) that intrinsic factor may possess species specificity. In this connection it is of interest to note that Toporek and his associates(14) found that, in contrast to the plateau effect of hog stomach preparations, progressively more Co^{60}-B_{12} was absorbed in pernicious anemia patients when normal human gastric juice was used as a source of intrinsic factor.

Conclusions. 1. The percentage excretion of radioactivity in the feces of normal and of totally gastrectomized rats, following administration of a single dose of 0.015 μg Co^{60}-B_{12}, was determined by scintillation counting. 2. The gastrectomized rats apparently were unable to absorb the labelled vit. B_{12} (100% fecal excretion). 3. Simultaneous administration of homogenized rat stomach or of rat gastric juice but *not* of human gastric juice or of an extract of hog stomach mucosa reproduced in the gastrectomized rat the capacity of the normal rat to absorb the test dose of Co^{60}-B_{12} administered (60% fecal excretion). 4. The rat, like man, secretes intrinsic factor largely, if not exclusively, into the stomach; but sources of human and hog intrinsic factor possess little or no activity in the rat. 5. The observations of Watson and Florey indicate that in the rat the secretion of intrinsic factor is probably confined to the distal secretory portion of the stomach.

The authors are grateful to Mrs. Rose B. Blumenthal and to Mrs. Ellen B. Block of the Thorndike Memorial Laboratory for technical assistance.

¶ Subsequently confirmed here in experiments upon 6 totally gastrectomized rats given ½ of the distal portion of a rat stomach after homogenization in water. The fecal excretions ranged from 54-72, average 60%, of the radioactivity administered. On the other hand, 5 similar experiments with the proximal portion of the rat stomach showed no effect upon fecal excretion: range 99-121, average 112%.

**Information kindly supplied by Dr. Kenneth W. Thompson of Organon Inc., Orange, N. J.

1. Berk, L., Castle, W. B., Welch, A. D., Heinle, R. W., Anker, R., and Epstein, M., *New England J. Med.,* 1948, v239, 911.

2. Heinle, R. W., Welch, A. D., Scharf, V., Meacham, G. C., and Prusoff, W. H., *Tr. A. Am. Phys.,* 1952, v65, 214.

3. Schilling, R. F., *J. Lab. and Clin. Med.,* 1953, v42, 860.

4. Glass, G. B. J., Boyd, L. J., Gellin, G. A., and Stephanson, L., *Arch. Biochem.,* 1954, v51, 251.

5. Swendseid, M. E., Halsted, J. A., and Libby, R. L., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 226.

6. Chaiet, L., Rosenblum, C., and Woodbury, D. T., *Science,* 1950, v111, 601.

7. Rosenblum, C., Woodbury, D. T., Gilfillan, E. W., and Emerson, G. A., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 268.

8. Prusoff, W. H., Welch, A. D., Heinle, R. W.,

and Meacham, G. C., *Blood,* 1953, v8, 491.

9. Chow, B. F., Quattlebaum, J. K., Jr., and Rosenblum, C., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 279.

10. Rosiere, C. E., and Grossman, M. I., *Science,* 1951, v113, 651.

11. Watson, G. M., and Florey, H. W., *Brit. J. Exp. Path.,* 1955, v36, 479.

12. Wallerstein, R. O., Harris, J. W., Schilling, R. F., and Castle, W. B., *J. Lab. and Clin. Med.,* 1953, v41, 363.

13. Callender, S. T., and Lajtha, L. G., *Blood,* 1951, v6, 1234.

14. Toporek, M., Bishop, R. C., Nelson, N. A., and Bethell, F. H., *J. Lab. and Clin. Med.,* 1955, v46, 665.

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Enzymatic Degradation of Side-Chain of Adrenocortical Steroids: Conversion of Hydrocortisone to Reichstein's E.* (22254)

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(Introduced by George Sayers.)

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In recent publications from this laboratory (1,2) an enzyme system found in both microsome and supernatant fractions of rat liver has been described which induces an alteration in the C-20 ketone group of adrenocortical steroids. Further observations on this system have been made and in addition positive evidence has been obtained that the reaction involving the C-20 ketone of the steroid molecule is reductive in nature.

Methods. Methods used in this study were identical with those described in previous publications (1,2).

Results. The first study in this series was undertaken to determine if the system reducing ring A could be separated from the system reducing the 20-ketone. A 0.25 *M* sucrose homogenate of rat liver free of nuclei and mitochondria was centrifuged at 20,000 X gravity for one hour. The supernatant fraction was discarded and the microsome fraction was resuspended and recentrifuged twice under the same conditions. Results obtained following incubation (37°C, 1 hr) of cortisone with the twice-washed microsome fraction are indicated in Fig. 1. It may be noted that the extent of Δ^4-3-ketone reduction in the complete system was only slightly less than that of 20-ketone reduction. Subsequent studies have confirmed this observation.‡ These results are at variance with those of Tomkins and Isselbacher(3) who reported that the Δ^4-3 ketone reducing system is present only in the supernatant fraction of rat liver.

p-Chloromercuribenzoic acid (PCMBA) inhibition of the Δ^4-3 ketone reducing system and of the 20-ketone reducing system in the microsome fraction was investigated. Fig. 2

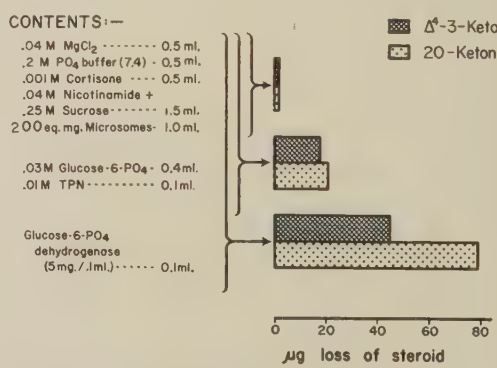

FIG. 1. Presence of both C-20 ketone and Δ^4-3-ketone reducing systems in microsome fraction of rat liver. See footnote (§) for definition of equivalent milligram (eq mg).

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† Data submitted in this report are in partial fulfillment for the Ph.D. degree. Predoctoral Fellow of the Arthritis and Rheumatism Foundation, 1955-1956. Present address: Upjohn Co., Kalamazoo, Mich.

‡ Recknagel, R. O., unpublished.

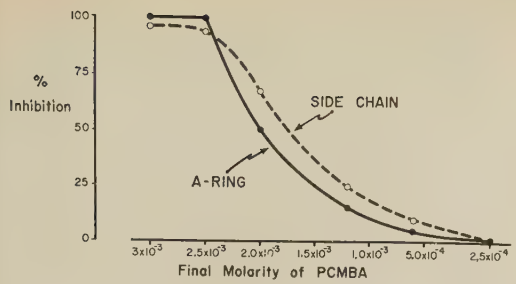

FIG. 2. Inhibition of Δ^4-3-ketone and C-20 ketone reducing systems by p-chloromercuribenzoate. Conditions as in Fig. 1.

indicates that inhibition of both systems was virtually complete at a final concentration of 3.0×10^{-3} *M* PCMBA. However, the inhibition by PCMBA appears to be due to inhibition of the TPNH generating system, since it was shown that glucose-6-phosphate dehydrogenase was strongly inhibited by PCMBA (Table I). The question of a dependence of C-20 and Δ^4-3-ketone reduction on SH-enzymes must therefore be considered as unsettled.

The enzymatic complexity of the microsome fraction with respect to reduction of steroid ketones is illustrated by the data of Fig. 3. In this study, equal quantities of cortisone, hydrocortisone, 11-desoxycortisone, tetrahydrocortisone, dehydroepiandrosterone, and androstenedione were incubated at 37°C for 2 hours using the conditions described in Fig. 1. Four-hundred equivalent milligrams§ of microsomes were used as source of enzyme. Loss of the C-20 group was followed by the Porter-Silber reaction(4). The Callow modification of the Zimmerman reaction(5) was

TABLE I. Inhibition of Glucose-6-Phosphate Dehydrogenase by p-Chloromercuribenzoate.

	E_{340}*
Complete system	.465
" " + 1×10^{-3} *M* PCMBA	.061
" " + 2 " "	.011
" " + 3 " "	.035

* Optical density reading at 340 mμ one min. after start of reaction.

Conditions: Sigma Chemical Co. Bulletin No. 201, Nov. 1951.

§ An equivalent mg of microsomes represents all of the microsome fraction obtained under prescribed centrifugation conditions from one mg wet weight of whole liver.

used to follow disappearance of the 17-ketone group. The steroids employed have a variety of biological actions. Tetrahydrocortisone and 11-desoxycortisone are biologically inactive. It is apparent that the metabolism of these steroids is not related in a simple and obvious way to their metabolic effects. The metabolism of typical 17 ketosteroids (dehydroepiandrosterone, androstenedione) in the microsome fraction adds to the complexity. The fact that tetrahydrocortisone reacts to a greater extent than the other steroids tested was confirmed by additional investigations (Fig. 4). During the first 90 minutes of in-

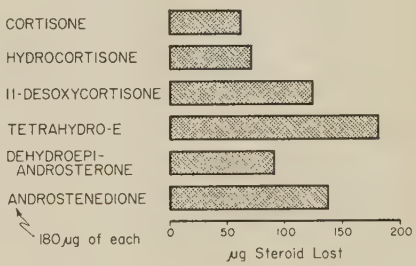

FIG. 3. Enzymatic complexity of rat liver microsomes with respect to reduction of steroid ketone groups.

cubation the loss of the 20-ketone of tetrahydrocortisone was about twice the rate observed for hydrocortisone. These *in vitro* observations reflect the *in vivo* observations of others, in which it was shown that tetrahydrocortisone disappears from the blood faster than hydrocortisone when administered to experimental subjects(6). Also, studies of Sandberg *et al.*(7) indicate that cortisone disappears at a more rapid rate from the blood than hydrocortisone, and that the steroid degradation products of hydrocortisone appear in the urine at a less rapid rate than the degradation products of cortisone. Whether the metabolic rate studies recorded in Fig. 4 represent the enzymatic basis for the *in vivo* observations cannot be stated with certainty at this time.

Isolation of Reichstein's E. Seven and two-tenths milligrams of hydrocortisone (free alcohol), dissolved in 20 ml of 0.25 *M* sucrose made to 2% with ethanol were incubated with 20 equivalent grams of rat liver microsomes (washed 2 times with 0.25 *M* sucrose). The

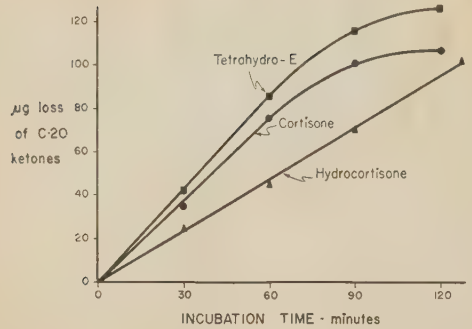

FIG. 4. Relative rates of reactions of various steroids in rat liver microsome fraction. Conditions as in Fig. 1.

following cofactors were added to the incubation medium: 14.0 ml of 0.03 *M* glucose-6-phosphate, 20.0 ml of 0.02 *M* phosphate buffer (pH 7.4), 10.0 ml of 0.04 *M* $MgCl_2$, 10.0 ml of 0.04 *M* isocitrate, 10.0 ml of 0.01 *M* TPN. The pH of the medium (7.4) was checked before incubation and 10.0 mg of glucose-6-phosphate dehydrogenase were added immediately before evacuation. The enzyme mixture was transferred to a 37°C water bath and incubated for 45 minutes. The aqueous phase was extracted 3 times with 400 ml $CHCl_3$. The $CHCl_3$ extract was dried under a stream of air and the residue redissolved in 70% ethanol and subsequently partitioned between 70% ethanol and pentane for removal of interfering substances. The pentane fraction was discarded and the 70% ethanol phase dried under vacuum. The dry residue was taken up in a small amount of chloroform and placed on a Florisil column (8). The column was eluted with 100 ml of chloroform, 100 ml of 2% methanol:chloroform, and 200 ml of 25% methanol:chloroform. The eluates were dried and the residues dissolved in methanol. One-twentieth of each eluate was applied to a paper strip and chromatographed in the benzene: 50% methanol:water system of Bush(9). Paper chromatograms were observed under ultraviolet light and subsequently sprayed with 4% ethanolic phosphomolybdic acid. Observation under the ultraviolet lamp revealed the presence in the 25% methanol-chloroform eluate of a compound more polar than hydrocortisone. The other eluates contained no detectable steroids. The phosphomolybdic acid reagent revealed (blue spot) the presence in this same eluate of a still more polar compound which was not visible under the ultraviolet lamp. In order to remove unreacted hydrocortisone the remainder of the 25% methanol-chloroform eluate was dried under vacuum and the dry residue placed on eight 1½-inch wide paper strips and chromatographed as before. The paper chromatograms were observed under the ultraviolet light. Regions which corresponded to hydrocortisone were discarded. More polar regions were eluted with methanol, dried under vacuum, redissolved, and a sample which corresponded to

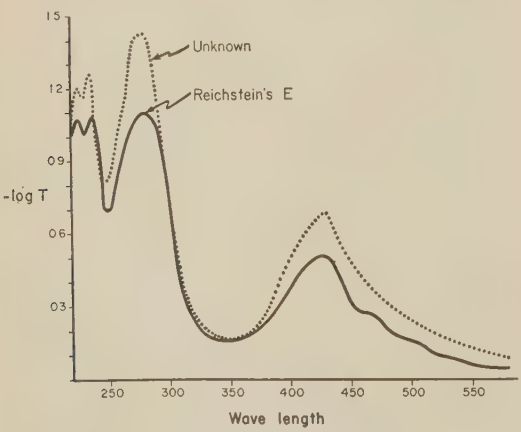

FIG. 5. Sulfuric acid induced absorption of unknown steroid compared to authentic sample of Reichstein's E.

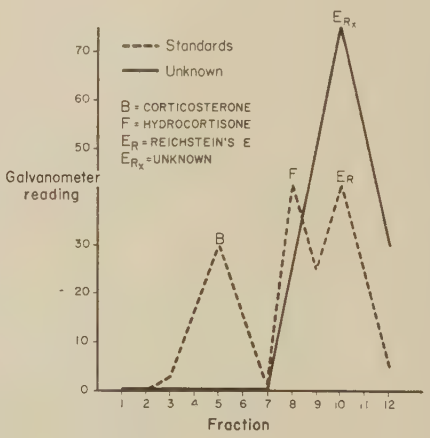

FIG. 6. Silica gel chromatography and fluorescence of unknown steroid compared to authentic sample of Reichstein's E.

one-twentieth of the original was rechromatographed in benzene:25% ethyl acetate. Ultraviolet and phosphomolybdic acid analysis revealed the presence of the 2 polar steroids as expected. In order to separate the 2 unknown steroids, the remainder of the methanol eluate was dried and rechromatographed on six 1½-inch wide paper strips in benzene:25% ethyl acetate. The unknown steroid which was not visible under the U-V lamp was not further analyzed. The areas on the 6 strips which were visible under the U-V lamp were eluted with methanol. In order to further purify the unknown steroid, the combined methanol eluates were rechromatographed on four 1½-inch wide paper strips in the benzene:25% ethyl acetate system. The ultraviolet absorbing steroid was eluted from the several strips, combined, dried under vacuum and diluted to 20.0 ml with absolute methanol.

The following tests with the recorded results indicate that the unknown compound was Δ^4-pregnene-11 β, 17 α, 20,21-tetrol-3-one (Reichstein's substance E). The stereochemical configuration of the C-20 alcohol of the experimentally derived product has not been definitively established.

Tests	Results
1. Rf values of unknown and known (free alcohols), on Bush(9) and Zaffaroni(10) systems.	1. Same Rf value for unknown and authentic sample of Reichstein's E.
2. Ultraviolet absorption spectrum at 220 to 260 mμ.	2. Absorption maximum at 240 mμ = Δ^4-3-ketone.
3. Phenylhydrazine reaction of Porter-Silber (4).	3. Unknown did not peak at 410 mμ. Not a C-20 ketone.
4. Sulfuric acid chromogen spectrum.	4. Spectrum (330 to 600 mμ). Identical with Reichstein's E. See Fig. 5.
5. Unknown chromatographed on silica gel column according to the method of Sweat(11).	5. Fluorescence and chromatographic behavior identical with Reichstein's E. See Fig. 6.
6. Unknown acetylated with pyridine-acetic anhydride 16 hr at room temp. Acetates of unknown and Reichstein's E chromatographed on Bush(9) and Zaffaroni(10) systems.	6. Rf values same for single chromatograms as well as mixed acetates.

Quantitative analysis at 240 mμ and calculation from known standards indicated that 2.1 mg of Reichstein's E were obtained from 7.2 mg of hydrocortisone. The more polar compound has not been definitely identified, but the evidence obtained suggests that it is a pentol. That the reactions in this system are probably exclusively reductive in nature was shown when 4-C^{14} hydrocortisone‖ was incubated with the preparation described, and the extracts chromatographed on the Zaffaroni system(10) for 92 hours. No C^{14} was present in the run-off from the paper strips. If oxidative reactions had occurred, 17-ketosteroids labelled with C^{14} would have been expected to appear in this fraction.

Conclusions. 1. Observations concerning the enzyme systems of the microsome fraction of rat liver which effect the C-20 ketone reduction of adrenocortical steroids have been described. 2. One product of hydrocortisone conversion in the microsome fraction of rat liver has been identified as Reichstein's E. The presence of at least one other product was noted.

We are indebted to Dr. John Schneider of the Jefferson Medical School for his generous gift of Reichstein's E, and to Dr. Constance de Courcy of the Jefferson Medical School for introducing us to the technics of the Bush system of paper chromatography.

1. Glenn, E. M., and Recknagel, R. O., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 153.

2. Recknagel, R. O., and Glenn, E. M., *ibid.*, 1955, v89, 156.

3. Tomkins, G., and Isselbacher, K. J., *J. Am. Chem. Soc.*, 1954, v76, 3100.

4. Porter, C. C., and Silber, R. H., *J. Biol. Chem.*,

‖ 4-C^{14}-Hydrocortisone obtained from U.S.P.H.S., courtesy of Dr. George Sayers. A Tracerlab Autoscaler, Model SC-51, and SC6C-Automatic Sample Changer were used for C^{14}-determinations.

1950, v185, 201.

5. Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, 1938, v32, 1312.

6. Brown, H., Willardson, D. G., Samuels, L. T., and Tyler, F. H., *J. Clin. Invest.*, 1954, v33, 1524.

7. Sandberg, A. A., Nelson, D. H., Glenn, E. M., Tyler, F. H., and Samuels, L. T., *J. Clin. Endocrinol. and Metab.*, 1953, v13, 1445.

8. Nelson, D. H., and Samuels, L. T., *ibid.*, 1952, v12, 519.

9. Bush, I. E., *Ciba Found. Colloquia*, 1955, 203.

10. Zaffaroni, A., and Burton, R. D., *J. Biol. Chem*, 1951, v193, 749.

11. Sweat, M. L., *Analytical Chem.*, 1954, v26, 1964.

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Steroid-Induced Adrenal-Pituitary Hypofunction II. (22255)

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It has been demonstrated that daily administration of hydrocortisone to rats lowers their tolerance to intraperitoneal injection of potassium chloride(1). Since this observation suggested adrenal-pituitary hypofunction, efforts were made to determine if these animals could be protected by treatment with various hormones. Intravenous administration of ACTH or aldosterone immediately prior to the injection of potassium chloride increased the tolerance of steroid-treated animals to KCl stress(2). It was observed that the same response was obtained if animals were given 1% NaCl to drink for 18 hours prior to injection of potassium chloride(3).

The protective action of sodium chloride suggested that the mortality of non-protected animals was not due to a hypofunctional adrenal or pituitary but rather to an electrolyte disturbance as a result of steroid treatment. The present experiments were designed (1) to measure the amount of sodium and potassium excreted in the urine during steroid administration, and (2) to determine the tolerance to potassium stress in rats depleted of sodium and potassium.

Methods. 1. Male rats, averaging 160 g body weight were placed in metabolism cages (2/cage) and fed Ingle's liquid medium carbohydrate diet *ad libitum*(4). Following a 2-week control period, these animals were injected once daily for 10 days with either 16% gelatin, 4 mg, hydrocortisone (F) in 16% gelatin, or 6 mg 11β, 17α-dihydroxy-4-pregnene-3,20-dione (21-desoxy-F) in CMC.* Urine volumes were recorded daily and urinary sodium and potassium were determined in a flame photometer. 2. Male rats were given intraperitoneal injections of 8 mg Thiomerin sodium (Wyeth), 5 mg Neohydrin (Lakeside), or 6 mg Diamox (American Cyanamid) in 0.2 cc aqueous solution. The rats were placed in metabolism cages, fasted for 18 hours and then injected with 0.16 M potassium chloride (5 cc/100 g body weight). The 18-hour urine collections were analyzed for sodium and potassium.

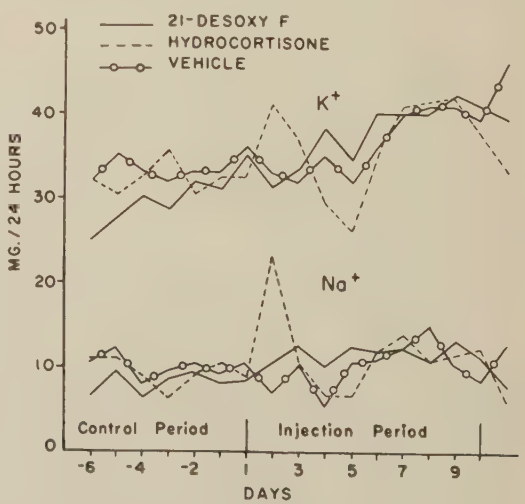

FIG. 1. Effects of hydrocortisone and 21-desoxy F on urinary sodium and potassium excretion.

* 0.5% carboxymethyl cellulose, 0.4% Tween 80, 1.5% benzyl alcohol and 0.9% sodium chloride.

TABLE I. Effects of Various Diuretics on Urinary Excretion of Sodium and Potassium.

No. rats	Treatment	% survivals*		Sodium, mg/18 hr	Potassium, mg/18 hr
49†	4 mg F/day/10 days	19	—	—	—
15	Controls	73	S‡ 11	10.4 ± 4.73	33.0 ± 3.20
			D 4	8.62± 3.87	34.6 ± 4.13
15	Thiomerin, 8 mg I.P.	73	S 11	13.5 ± 3.87	37.7 ± 7.01
			D 4	10.37± 2.80	35.75± 1.69
15	Neohydrin, 5 mg I.P.	53	S 8	14.3 ± 4.85	40.7 ± 9.03
			D 7	15.5 ± 7.09	43.8 ± 9.56
15	Diamox, 6 mg I.P.	66	S 10	23.8 ± 6.13	43.9 ± 8.73
			D 5	24.6 ± 6.63	42.0 ± 9.54

* Stressed with 0.16 M KCl, 5 cc/100 g body wt. † Collins(1). ‡ S = Survivals; D = Deaths.

Results. Fig. 1 demonstrates the pattern of sodium and potassium excretion during daily administration of F or 21-desoxy-F for 10 days. Urinary excretion of sodium and potassium increased during the 24-hour period following the injection of F. This increased excretion was not maintained. The animals showed a decrease in the excretion of both sodium and potassium during the third day of treatment. Decreased urinary excretion of sodium and potassium coincided with a period of decreased food intake. By the sixth day, food intake was again normal and urinary electrolyte concentrations paralleled that of control animals.

Animals injected with 21-desoxy-F demonstrated a more gradual and sustained increase in sodium and potassium excretion. However, the increased urinary concentration of these ions was not too different from the values observed in control animals.

Table I gives the effects of various diuretics on the urinary excretion of sodium and potassium as well as the results following potassium stress. Urinary excretion of sodium and potassium in the Diamox-treated group is significantly higher than the control group. However, within any given group, there is no significant difference between the animals that survived the stress and those rats in which the potassium chloride proved fatal. Fifty-three per cent of the Neohydrin-treated animals survived after potassium stress. However, this survival rate is higher than the survival rate for hydrocortisone-treated animals.

Discussion. Dietary potassium enters the blood stream and becomes available to the cells via the extracellular fluid. When potassium is presented to the cells, the amount taken up is dependent upon cellular requirements. If the supply exceeds cellular demands, the excess potassium is presumably eliminated by renal excretory mechanisms. Increased concentrations of extracellular sodium dilutes extracellular potassium and increases renal elimination of potassium which results in a loss of intracellular potassium. These actions of sodium make it useful in the treatment of potassium intoxication. Conversely, depletion of sodium enhances the possibility of potassium intoxication(5-7).

Although the mechanism of potassium stress in animals with hypofunctional adrenal cortices has not been defined, a hydrocortisone-induced increase of sodium excretion is suggestive of a decreased tolerance to a potassium load. However, the present data indicate that marked sodium depletion does not decrease the resistance of rats to potassium stress if adrenal-pituitary function is not impaired. The sodium losing effect of hydrocortisone is of 1-3 days duration and is not sustained during chronic administration. This is in agreement with the observations of Ingle *et al.*(8). The data which have been presented above indicate that decreased resistance to a potassium load results from a decrease in adrenal-pituitary function and not from steroid-induced changes in electrolyte composition of the organism.

Summary. Data are presented which in-

dicate that marked sodium depletion does not alter the resistance of intact rats to a potassium load. Early but transitory urinary electrolyte changes were observed during chronic hydrocortisone treatment. The decreased resistance to potassium stress in animals treated with hydrocortisone is probably due to hypofunction of the adrenal-pituitary axis rather than to steroid-induced alterations of electrolyte balance.

1. Collins, E. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 443.

2. ———, 1955, in press.

3. ———, unpublished data.

4. Stafford, R. O., Bowman, B. J., and Olson, K. J., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 322.

5. Merrill, J. P., Levine, H. D., Somerville, W., and Smith, S., *Ann. Int. Med.*, 1950, v33, 797.

6. Finch, C. A., Sawyer, C. G., and Flynn, J. J., *Am. J. Med.*, 1946, v1, 337.

7. Luetscher, J. A., Ciba Foundation, *Colloquia*, 1952, vIV, 430.

8. Ingle, D. J., Sheppard, R., Oberle, E. A., and Kuizenga, M. H., *Endocrinology*, 1946, v39, 52.

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Effect of Choline, Heparin and Aureomycin on Fatty Livers of Dogs.* (22256)

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Gyorgy, *et al.*(1,2) demonstrated that Aureomycin exerts a lipotropic and anti-cirrhotic effect in rats fed a high fat, low protein, choline deficient diet. Baxter and Campbell (3) noted prevention of severe renal injury and some diminution of liver fat after Aureomycin therapy. More recently the amount of fat in livers has been reduced in cholesterol-fed rabbits(4) and rats(5) by the daily administration of heparin. It therefore became of interest to compare the effectiveness of choline, Aureomycin and heparin in a different species, the dog, and to ascertain whether the mechanism of lipotropic action of Aureomycin was related to the choline sparing effect of this drug(3,6).

Methods. Healthy mongrel dogs, previously maintained on Purina laboratory chow were fed 14 g per kg body weight of a high-fat, low-protein, choline-deficient diet‡(7) for 21 days. Eight of these animals received 0.5 g of Aureomycin§ (Chlortetracycline) in capsule form in the morning and late afternoon simultaneously with the feeding of the diet. Seven animals received daily oral supplements of 1% choline chloride.‖ Nine other animals were injected twice a day with heparin§ 2 mg/kg body weight intramuscularly. On the 21st day, some animals were killed by rapid intracardiac air injections and liver slices were made by a Martin slicer(8). Liver slices were incubated with radioactive phosphate for one hour, with and without the *in vitro* addition of choline chloride (1 mg/cc of medium). Composition of medium and isolation of phospholipides have been described(6). From the specific activity of the inorganic phosphate of the medium and the total radioactivity in the phospholipide, the *in vitro* rate of phosphatide synthesis was calculated. The remaining dogs were sacrificed after the 3 week experimental period. Phospholipide phosphorus was determined on an

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‡ The diet was composed of 38% lard, 44% sucrose, 8% vitamin test casein, 3% cod liver oil and 5% Cowgill's salt mixture. Any animal that did not consume entire diet upon feeding was force-fed.

§ Kindly donated by Lederle Co., Pearl River, N. Y.

‖ Kindly donated by Merck and Co.

TABLE I. Effect of Choline, Heparin and Aureomycin on Liver Lipide Partition.*

Group	No. animals	Phosphatide	Total cholesterol	Total lipide	Triglyceride + cholesterol ester fatty acid
Normal	7	29.4 ± 1.9	2.43 ± .13	43.1 ± 2.1	12.4 ± 1.7
HF†	11	20.1 ± 1.2	2.68 ± .14	64.7 ± 5.2	42.0 ± 4.0
" + choline	7	21.5 ± 1.6	2.32 ± .22	35.9 ± 2.4	12.2 ± 1.1
" + aureomycin	8	17.1 ± 1.2	2.92 ± .22	94.5 ±14.1	73.3 ±13.9
" + heparin	9	16.9 ± 1.1	2.46 ± .30	47.6 ± 3.5	28.3 ± 4.1

* Lipide values expressed as mg/g fresh tissue wt (mean ± stand. error).
† HF = High fat.

alcohol-ether, petroleum ether extract of liver (9). Total cholesterol was analyzed by the method of Sperry and Webb(10) and total lipides were gravimetrically determined by evaporating an aliquot of the petroleum ether extract. The difference between the total lipide on the one hand and the amount of phospholipide (lipide P x 25) plus cholesterol on the other was designated as triglyceride plus cholesterol-ester fatty acid fraction.

Results. The lipide partition in the livers of dogs on the various regimens is presented in Table I. The liver phosphatide concentration of animals on laboratory chow was significantly greater than that in any of the other groups. Neither treatment with heparin or Aureomycin, nor addition of daily adequate choline supplements had any effect on the depressed phosphatide levels of dogs maintained on the high-fat diets. Thus it appears that the decrease in liver phosphatides of dogs on the basic high-fat, low-protein diet was not the result of a simple choline deficiency but may be related to absence of adequate amino acids or other factors from the diet.

In contrast to the decrease in liver phosphatides, the cholesterol concentration was remarkably constant in all animals. The greatest alterations occurred in the "triglyceride" fraction (last column of Table I). Since the total cholesterol did not differ greatly from one group to another and constituted only a small fraction of total liver lipides, it is reasonable to assume that the different values in the last column of Table I primarily reflect alterations in triglyceride concentrations. Thus we may conclude that supplementing the high fat diet with 1% choline during the 3 week experimental period maintained triglycerides at normal concentrations. Administration of heparin to dogs on the high-fat diet appeared to exert some lipotropic effect since lowering of liver triglycerides was statistically significant at the 3% level of confidence. Aureomycin, on the other hand, did not exert any lipotropic action. Quite to the contrary, it appeared that triglyceride levels in Aureomycin treated animals exceeded those of the untreated controls on the same diet ($P = 2.5\%$).

From these experiments one might conclude that Aureomycin aggravated the degree of choline deficiency, but the next set of experiments throws additional light on these results. Table II summarizes data on the rate of phosphatide synthesis by liver slices incubated with radioactive phosphate in the presence and absence of choline in the medium. The first 3 rows confirm our earlier findings (6) that *in vitro* addition of choline markedly stimulates formation of P^{32} labeled phospholipides in choline-deficient liver slices, but has practically no effect on liver slices from dogs maintained on Purina laboratory chow or an high-fat, low protein diet supplemented with 1% choline. If the high triglyceride content of livers of Aureomycin-treated dogs was the result of a choline deficiency similar to that of the animals on unsupplemented high-fat diet (second row of Table II) one would expect a marked increase in phosphatide synthesis of slices in choline supplemented baths. That this is not the case may easily be seen from the figures in the bottom row of Table II. Slices incubated in choline supplemented medium showed a slightly greater synthesis of radioactive phospholipides than slices incubated without choline, but the difference did not compare to the

TABLE II. Phospholipide Concentrations* and Rates of Synthesis in Liver Slices† with and without Added Choline.

Group	mg phospholipide P/g		γ P converted to phospholipide/hr/g	
	Without choline	Choline added	Without choline	Choline added
Normal	.93 ± .052	.99 ± .044	11.8 ± 2.5	12.9 ± 1.2
HF‡	.60 ± .049	.68 ± .063	9.5 ± .7	21.9 ± 2.3
" + choline	.73 ± .060	.75 ± .056	8.6 ± .3	9.7 ± .9
" + aureomycin	.57 ± .073	.58 ± .053	10.8 ± 2.0	14.2 ± 2.1

* Concentrations expressed as mg of phospholipide phosphorus/g fresh liver. Values are given as mean of 4 dogs ± stand. error.

† Each animal liver furnished 6 slices; 3 incubated with choline, 3 without; one slice/flask; each slice was analyzed separately.

‡ HF = High fat.

nearly 2½ fold increase observed in animals with known choline deficiency. According to this test, therefore, Aureomycin decreased the severity of choline deficiency. The inability of Aureomycin to influence the rate of phospholipide synthesis of liver slices agrees with the report of Cornatzer and Gallo(11) that this antibiotic had no effect on phospholipide turnover in the liver of intact rats.

Discussion. It might be well to emphasize that our results were obtained on adult dogs maintained on a high fat, high carbohydrate diet, deficient in choline and methionine, and possibly also in other unknown factors. The fact that addition of choline to our basal ration completely restored the normal appearance and normal triglyceride content of livers should not obscure the fact that the choline supplement failed to restore normal liver phospholipide levels. As a matter of fact, this finding constitutes perhaps one of the most interesting aspects of our study, namely, that the frequently observed parallelism between the lipotropic effect of choline and its effect on liver phospholipide metabolism may not be a cause and effect relationship, but may be more of a coincidental phenomenon. In the present study, for example, the *daily additions* of choline to high-fat diet exerted a marked lipotropic effect but failed to raise the liver phospholipide concentration or the rate of phospholipide synthesis in the liver slice (8.6 γ/g/hr as compared to 9.5 γ/g/hr). This finding is difficult to reconcile with the well known phospholipide-turnover-stimulating action of a single dose of choline given to choline-deficient intact animals(12), or added to their liver slices(6,12).

Gyorgy(2) employing a diet similar to ours observed that in rats Aureomycin markedly decreased liver lipide concentrations. Kaplan *et al.*(13) employing blood lipide alterations as his criterion concluded that in pancreatic duct-ligated dogs, Aureomycin exerted some lipotropic activity. Some choline sparing action appeared to occur in our animals as demonstrated by the failure of the liver slice to respond to added choline. However, this choline made available by Aureomycin was not effective in lowering liver lipides. Since large doses of Aureomycin have been reported to cause fatty livers in mice(14), we fed 1 g of Aureomycin per day for 3 weeks to 2 dogs maintained on Purina laboratory chow without any visible or chemical effects on liver lipides. Similar results were reported by Kaplan *et al.*(13) and Sutherland *et al.* (15). Thus, administration of this amount of Aureomycin to dogs on a nutritionally adequate diet was not associated with fatty liver development. Under the conditions of our experiments Aureomycin apparently exerted some choline sparing action without a concomitant lipotropic effect on the liver, but such an hypothesis deserves further studies.

Summary. The triglyceride concentration in livers of dogs maintained for 3 weeks on a high-fat, low-protein, choline-deficient diet was lowered by daily injections of heparin and by supplementation of the diet with choline. Oral administration of Aureomycin increased liver triglyceride concentrations. Choline, heparin or Aureomycin did not alter liver phosphatide or cholesterol concentra-

tions of animals receiving the high-fat diet. Aureomycin, *per os,* did not affect *in vitro* synthesis of liver phosphatides, but modified the response of liver slices to the *in vitro* addition of choline.

1. Gyorgy, P., Stokes, J., Jr., Goldblatt, H., and Popper, H., *J. Exp. Med.,* 1951, v93, 513.

2. Gyorgy, P., *Ann. N. Y. Acad. Sci.,* 1954, v57, 925.

3. Baxter, J. H., and Campbell, H., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 415.

4. Meng, H. C., and Davis, W. S., *A.M.A. Arch. Path.,* 1955, v60, 276.

5. Kessler, W. B., and Meng, H. C., *Fed. Proc.,* 1954, v13, 78.

6. Di Luzio, N. R. and Zilversmit, D. B., *J. Biol. Chem.,* 1953, v205, 867.

7. Entenman, C., Chaikoff, I. L., and Friedlander, H. D., *ibid.,* 1946, v162, 111.

8. Martin, A. W., *Endocrinology,* 1942, v30, 624.

9. Zilversmit, D. B., and Di Luzio, N. R., *J. Biol. Chem.,* 1952, v194, 673.

10. Sperry, W. M., and Webb, M. J., *ibid.,* 1950, v187, 97.

11. Cornatzer, W. E., and Gallo, D. G., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 15.

12. Artom, C., *Liver Injury,* Hoffbauer, F. W., Editor. Trans. 10th Conf. New York, Josiah Macy, Jr. Foundation 62, 1951.

13. Kaplan, A., Cohn, C., Narahara, A., and Jones, K., *Ann. N. Y. Acad. Sci.,* 1954, v57, 688.

14. Lepper, M. H., Zimmerman, H. J., Carroll, G., Caldwell, E. R., Jr., Spies, H. W., Wolfe, C. K., and Dowling, H. F., *A.M.A. Arch. Int. Med.,* 1951, v88, 284.

15. Sutherland, D. A., Mann, J. D., Giges, B., and Seligson, D., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 458.

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Rapid Procedure for Erythrocyte Packed Cell Volume and Sedimentation Rate Determinations. (22257)

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During laboratory investigations involving large numbers of human blood samples, erythrocyte sedimentation rates and packed red cell hematocrit values were required for correlation with other observations. This need stimulated efforts to develop time saving modifications of the conventional procedures primarily to devise a rapid preliminary screening operation for selection of abnormal samples for further study by standard methods. However, in actual practice the rapid procedure yielded accuracies sufficiently comparable to standard methods that its routine substitution for the more time-consuming technics appeared possible. The procedure was put on a quantitative basis and appropriate comparison studies with accepted methods(1,2) were carried out. Results here presented indicate that the values are satisfactory for routine clinical use and that they may be obtained with substantial time saving and other economies. The method has been used in testing over 10,000 patient blood samples. The essence of the modification consists in determining sedimentation rate and hematocrit values directly in the original blood collection tube without necessity of transfer to a secondary, calibrated hematocrit tube. The recent introduction of rubber stoppered, evacuated, blood collection tubes containing appropriate anti-coagulants contributes to the speed and convenience of this technic. However, any standard test-tube or collection tube may be substituted or adapted to this procedure. A basic problem of this modification, the variable amount of blood sample in each collection tube, has been overcome by use of a proportional volume chart accurately indicating percentage of relative parts of a test tube blood sample irrespective of its total volume (Figs. 1 and 2). The

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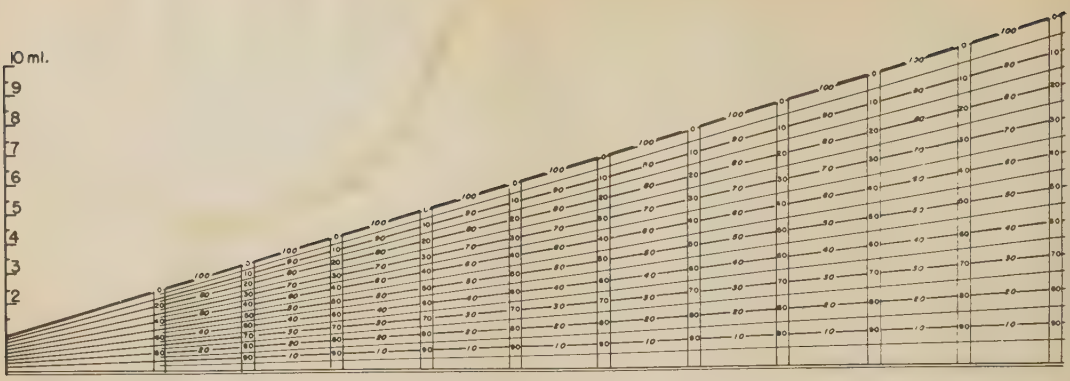

FIG. 1. Proportional volume chart for determining relative percentage of separated substances in an uncalibrated tube irrespective of volume fluctuations.

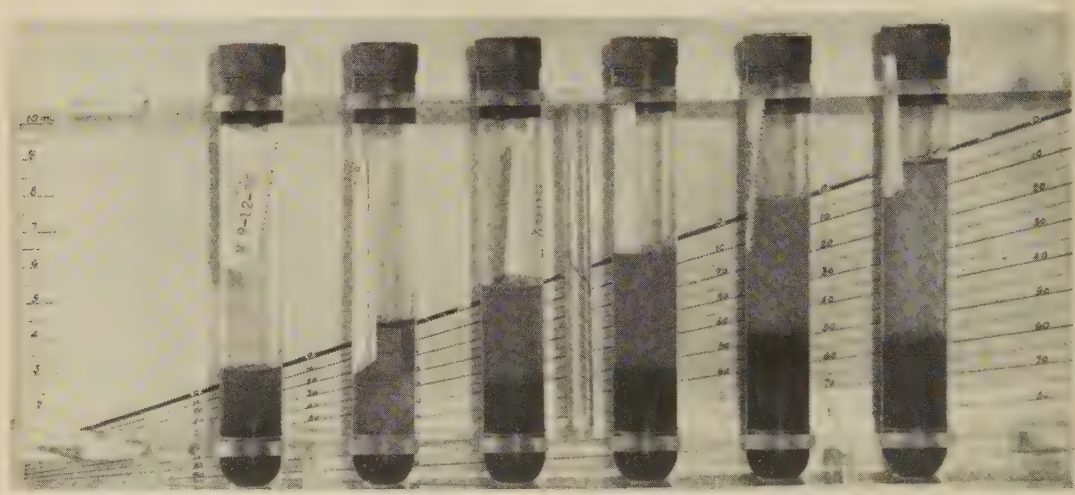

FIG. 2. Blood collection tubes arranged to illustrate use of chart in determining sedimentation rates or hematocrit values irrespective of sample volumes.

design of the chart takes into consideration corrections necessary for the spherical bottom of the tube, the diameter, shape and thickness of glass, and the meniscus. The chart (Fig. 1) was designed for standard blood collection tubes† used in our studies but, as can be readily shown, a reasonable degree of variation in these factors can be tolerated.

Method. Erythrocyte sedimentation rate. Blood is collected by standard venepuncture procedure, employing a syringe and test tube with suitable anticoagulant, or the commercially available rubber-stoppered Vacutainer† tube used with double-ended needle and holder. These studies employed the evacuated tubes containing either heparin or oxalate. The sealed samples are numbered, or otherwise identified, and placed in racks suitable for holding the tubes perfectly perpendicular (Fig. 2).‡ An optional procedure found useful in sharpening the plasma meniscus is the addition of silicone anti-foam emulsion.§ A tiny drop is added to each tube with a No. 24 needle and a one-ml tuberculin syringe. The blood is mixed thoroughly by inverting the entire rack vigorously 10 times, and is then

† The Becton, Dickinson, and Company Vacutainer blood collection tubes containing laboratory grade heparin or mixture of dry ammonium and potassium oxalate.

‡ Chart, racks, and accessory equipment manufactured by Virtis Co., Yonkers, N. Y.

§ Dow Corning antifoam A emulsion, a silicone defoamer.

placed on a level vibrationless surface to sediment for 30 minutes. If more than one rack is run simultaneously, the mixing interval between racks should correspond to the rack reading time of the technician. This interval is approximately one minute with a 10-tube rack for an experienced operator. If a time course curve is desired, appropriate interval readings may be taken, provided tube contents are not unduly disturbed. At termination of the sedimentation period, relative rates are read by placing the rack of tubes in front of the reading chart and sliding the entire rack to the left or right as required to cause the top of the blood sample of a given tube to coincide with the upper heavy meniscus line of the chart. The sedimentation rate of the red cells is then read from the appropriate chart line by noting the position on the chart of the erythrocyte-plasma interface. Either edge of tube may be employed in making the reading but proper eye level should be carefully observed to avoid parallax errors. It will be noted that the chart has 2 reading scales. The numbers reading from top down are for red-cell sedimentation rate, while those reading from bottom up refer to packed red cell volume. If repeat determinations are desired,

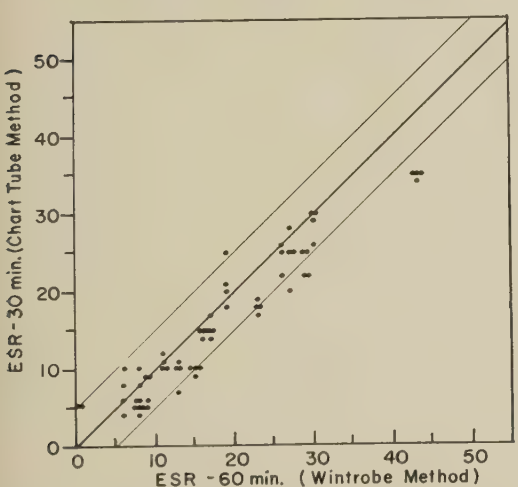

FIG. 3. Values obtained throughout the erythrocyte sedimentation rate range with tube-chart procedure as compared with Wintrobe technic. (Tube values are plotted about the straight line representing Wintrobe values. Scattering reflects combined errors of both methods. Boundary lines equal plus or minus 5 reading points.)

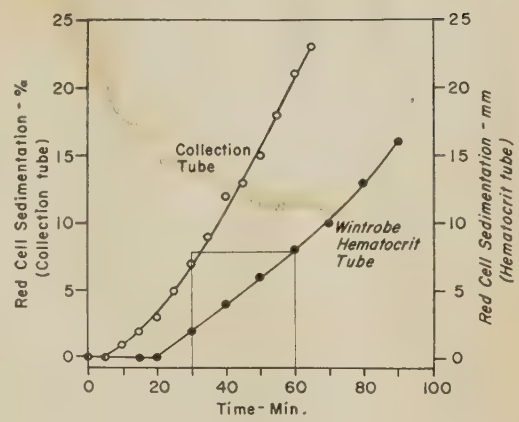

FIG. 4. Comparison of time-course curves of same blood sample determined by tube-chart and Wintrobe procedures.

the entire rack or a given sample may be remixed and reread as many times as required, the only limitation being possible changes in sedimentation rate with time. *Packed red cell volume.* The hematocrit value, or red cell volume, is obtained by transferring the blood collection tubes to a suitable centrifuge and spinning for 10 minutes at 2000 rpm (mean RCF = approximately 700). The large, 4-place horizontal head, No. 284 or 289, of the standard International centrifuge is convenient since each cup will handle from 8 to 12 tubes depending upon diameter of tube used, permitting a total load of 32 to 48 blood samples. Care should be taken in packing the tubes that they are as nearly vertical as possible so that the packed red cell interface will be level in the tube for optimum accuracy in reading. At completion of the run, hematocrit values are read in the same manner as sedimentation rates except that numbers running from bottom of chart upward apply. The percentage of white cells and platelets, or buffy coat, are noted in the usual fashion and their percentage may be read from the chart. Icterus or other abnormalities of the plasma may be recorded as hematocrits are read.

Results. Erythrocyte sedimentation rate. A comparison of results between this procedure and the conventional Wintrobe method is illustrated in Fig. 3 and 4. In Fig. 3, values for a group of blood samples covering a wide range of sedimentation rates are plotted to illustrate normal scattering of points obtained

by this method at all levels of sedimentation rate when referred to Wintrobe values. This was done by employing a standard time factor of 30 minutes in the case of the tube-chart method as compared with 60 minutes with the Wintrobe hematocrit tube. It may be seen that over 80% of the points fall within the plus or minus 5% boundaries established by the standard Wintrobe procedure on the same blood samples. It will be remembered that in this type of graphic plot the combined errors of both methods are reflected in the scattered points about the line. The aberrant points thus may be due to an excessive error by either method or to the smaller combined errors in the same direction by both methods.

Fig. 4 shows the time course plot of the same blood sample when run by the 2 methods. Differences in slope of rate curves illustrate the basis for approximate equivalence of 30- and 60-minute readings for the 2 methods. It is seen that the vertical lines at these 2 time periods intersect the sedimentation curves at approximately the same reading level. This slope increase has the advantage of shortening the time requirement for sedimentation to reach a desirable measuring point, and the disadvantage of increasing the critical importance of the time factor. Thus a lag of 2 minutes in reading the ESR by the tube-chart method would cause a greater error than the same lag period with the Wintrobe hematocrit. Although 30-minute and 60-minute readings are not always equivalent they are statistically close enough to take advantage of the convenience of the 30-minute time period.

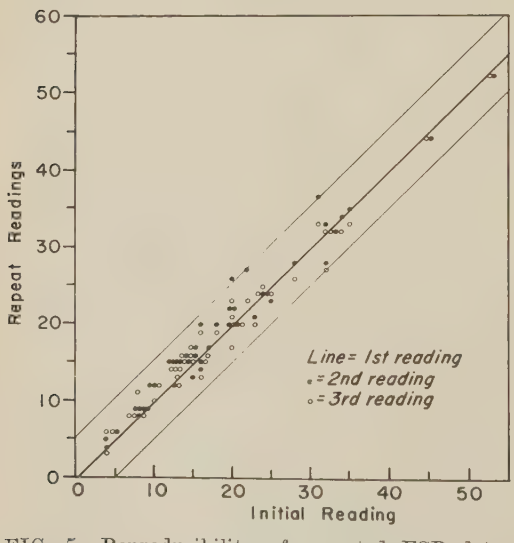

FIG. 5. Reproducibility of repeated ESR determinations over a wide range employing tube-chart technic.

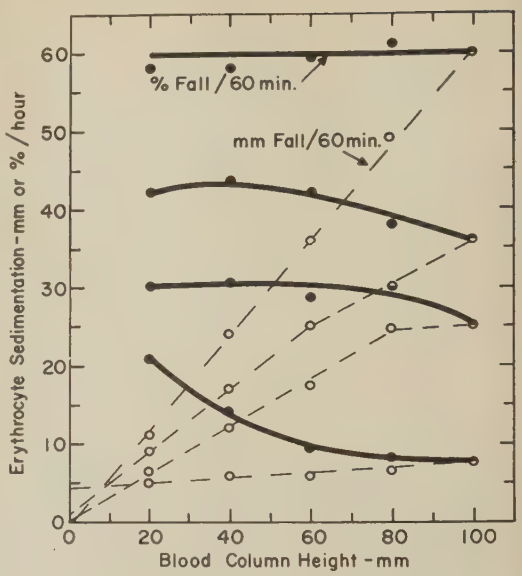

FIG. 6. Comparison of mm and percentage sedimentation rate readings employing identical blood samples with different column heights. Four levels of sedimentation rate are illustrated. Solid lines = percentage sedimentation rate (% of total height /60 min). Broken lines = mm of red cell sedimentation/60 min.

Reproducibility of this procedure with repeat determinations on the same blood samples over a wide ESR range is illustrated in Fig. 5. The original readings are plotted as the diagonal reference line and subsequent second and third readings are plotted as the closed and open circles. The moderate degree of scattering throughout the entire range indicates that reproducibility is not biased by magnitude of the sedimentation rate.

The published procedures for measuring erythrocyte sedimentation rate employ "millimeters" of fall of the top boundary of the settling red cell population. The several technics in current clinical use employ hematocrit tubes of varying lengths fluctuating from 40 to 200 mm(2-5). Since the procedure reported here employs "percentage" of fall rather than absolute distance in mm it was necessary to investigate the relationship between abso-

lute and percentage measurements within the anticipated extremes of sample volume fluctuation and thus of blood column height. Under conditions of our testing operations the extremes of whole blood sample volume gave column heights of 20 to 80 mm with the preponderance of samples from 50 to 70 mm in height. Fig. 6 illustrates the relationship between absolute mm of fall and percentage, or relative fall, when testing identical blood samples of different height. The data also show the relationship between blood samples with widely differing sedimentation rates.

For all sedimentation rate levels except the lowest one, the blood column height has little influence on percentage fall, whereas there are substantial differences in the reading of various height tubes when mm are the criteria of measurement. This accounts for some of the numerical confusion in attempting to establish normal and abnormal standards of sedimentation rate between several methods utilizing different column heights(2-7). With the percentage technic described here the sample column height is not critical between the range of 20 to 100 mm unless the ESR is low, in which case samples less than 40 mm in height should be avoided, corrected, or transferred to a smaller bore tube which will increase the sample height to 50 or 60 mm.

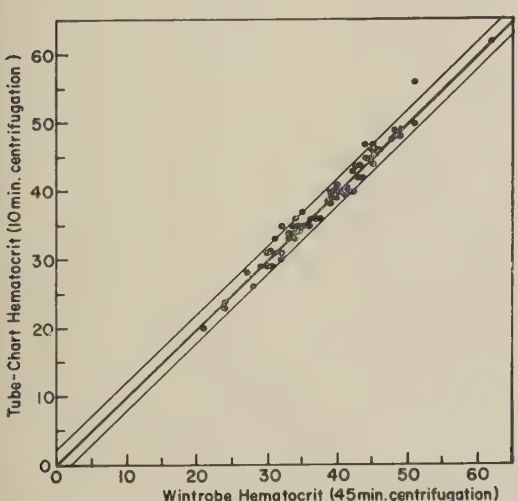

FIG. 7. Comparison of hematocrit values on 68 blood samples determined by both tube-chart and Wintrobe procedure employing 10 and 45 min. centrifugation respectively. (rcf = 700, rpm = 2,000.)

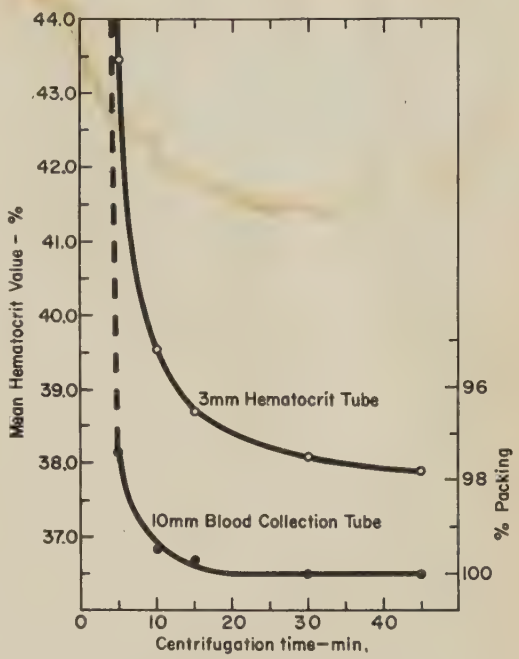

FIG. 8. Comparison of sedimentation curves in centrifugal packing of red cells when employing a 13 mm blood collection tube and a 3 mm hematocrit tube. These curves are mean values of 20 samples run simultaneously from the same group of standard hospital blood samples.

The foregoing data illustrate that the expression of erythrocyte sedimentation by percentage fall is simpler and more uniform than arbitrary sedimentation rate in mm for columns of varying heights which will yield strikingly different values for the same blood sample depending upon the style of tube employed. None of the ESR values reported here are corrected for differences in packed red cell volume although this correction does not significantly alter the relationships illustrated. The standard Wintrobe chart can be used for this purpose with this method in the same way as employed for the Wintrobe values.

Red cell packed volume. A comparison of this procedure and the Wintrobe in hematocrit determinations on 68 samples of blood covering a wide range of values is illustrated in Fig. 7. It is seen that the 2 methods agree very closely and that 95% of the points lie within the plus or minus 2% boundaries. The

difference in required centrifugation time between the 2 methods is shown in Fig. 8. It may be noted that the stationary packing point for a given relative centrifugal force is reached much more rapidly in the larger diameter collection tube and that 5 minutes of centrifugation at 2000 rpm (rcf = 700) is equivalent to the packing obtained in about 30 minutes with the narrow bore hematocrit tube. After 10 minutes of centrifugation the packing value is about 1 or 2% greater than the Wintrobe tube after 45 minutes of centrifugation.

Discussion. The advantages of this method are principally convenience, speed, and economy. Relative accuracy is indicated by Fig. 3, 5, 6, and 7 which illustrate that the obtained values are comparable with a standard current method and distinguish the normal and abnormal categories in the same quantitative manner.

A beneficial result derived from the use of relatively large diameter collection tubes of 10 to 15 mm as compared with 3 mm bore Wintrobe tubes is the substantial increase in speed of the red cell sedimentation. The erythrocyte sedimentation rate is approximately doubled, making it possible to reduce the sedimentation period by one-half, *i.e.,* from 60 to 30 minutes (Fig. 3, 4). The same advantage may be exploited in the packed red cell determination (Fig. 8) where the hematocrit value in the collection tube after 5 minutes of centrifugation is approximately equivalent to the standard Wintrobe procedure at 30 minutes. As a consequence of the various economies, a single technician can run sedimentation rate or hematocrit determinations on several hundred patient blood samples per day. In addition to modification of technical procedure, this method introduces and utilizes the concept of presenting sedimentation rates, as well as hematocrits, as percentages rather than absolute millimeters of fall as commonly expressed (Fig. 6). In the case of the 100 mm Wintrobe hematocrit tube, both mm and percentage criteria are the same, of course.

Since there is no necessity for the transfer of an accurate representative sample, as there is in the usual determination, the danger of sampling error due to improper mixing or measuring is avoided. The additional safety factor of handling a sealed container when testing bloods which may contain infectious agents is a distinct advantage and protection for laboratory personnel. With the use of disposable test tubes there is, of course, no glass washing or hematocrit tube cleaning problem.

Finally, since the blood sample is not used up or contaminated it is available for all other routine determinations which can utilize plasma or whole blood. Bacteriological studies, cellular counts, and many of the blood chemistry determinations may be made from the one blood sample tube(1,6).

Under some circumstances a rapid screening of blood samples for abnormalities is useful. A high degree of accuracy in spotting abnormal specimens may be obtained by taking a 15-minute reading. Values exceeding 10 should be considered abnormal. Final readings should, of course, be made at 30 minutes for confirmation and to obtain standard comparative values.

The proportional volume chart and procedure may also be used for other purposes such as measuring the relative percent of cells in ascitic fluid, the various percentile components of a centrifuged homogenate, sedimented bacteria, chlorella, etc., with no concern for a precise sample volume or calibrated tubes.

Summary. The conventional erythrocyte sedimentation rate and the packed red cell volume procedures have been modified to effect more than a 50% reduction in the operational time required. The essence of the modification consists in determining the sedimentation rates and hematocrit values directly in the original blood collection tube without the necessity of the usual transfer to a secondary, calibrated hematocrit tube. This is accomplished by employing a proportional volume chart which gives the sedimentation rate or hematocrit value for the red cells irrespective of the sample volume fluctuations. All values are expressed as percentages rather than the absolute measurements commonly employed. Erythrocyte sedimentation time is reduced from the standard of 60 to 30 minutes, and hematocrit centrifugation time is re-

duced from 30 to 10 minutes to yield approximately equivalent values. The method has been successfully tested with over 10,000 patient blood samples. The procedure may also be used for similar proportional measurements with ascitic fluid, tissue homogenates, bacterial suspensions, etc., with no need for exact sample volume or calibrated tubes.

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1. Wintrobe, M. M., *Am. J. Med. Sci.*, 1933, v185, 58.

2. Wintrobe, M. M., and Landsberg, J. W., *ibid.*, 1935, v189, 102.

3. Cutler, J. W., *J. Lab. and Clin. Med.*, 1940, v26, 542.

4. Cutler, J. W., *Am. Rev. Tuberc.*, 1943, v48, 314.

5. Westergren, A., *Ergebn. d. inn. Med. u. Kinderh.*, 1924, v26, 577.

6. Hadley, G. G., and Larson, N. L., *Am. J. Clin. Path.*, 1953, v23, 6.

7. Queries to Editor, *J. Amer. Med. Assoc.*, 1955, v149, 1422.

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Autoradiography of Carbon-14 Labeled Isoniazid in Brain.* (22258)

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The widespread interest in drugs affecting the central nervous system, together with the increasing numbers of isotopically labeled drugs available in research, has stimulated the search for methods of producing autoradiograms of nervous tissue containing compounds labeled with such soft beta emitters as C-14. To date no suitable method has been developed for preparing brain autoradiograms for C-14 containing specimens. Previous methods have been described showing the localization of certain other isotopes not only in individual cells but even in inclusion bodies(1,2). These methods have not, however, been applied to such soft tissues as brain.

The autoradiograms described in this paper, together with supporting differential counting data, show the localization of C-14 labeled isonicotinic acid hydrazide (Isoniazid) and/or its metabolites in gross anatomical structures of brains. C-14 labeled Isoniazid was used as a prototype for these studies because of its known central nervous system effects and because it has previously been shown to localize in the nervous system of mice, rats, and guinea pigs(3). These observations on the localization of C-14 labeled Isoniazid have been confirmed by us in rats and cats, which were used in the autoradiograph studies.

Materials and methods. The Isoniazid used in these studies was synthesized by Murray and Langham of the Health Division, Los Alamos Scientific Laboratories(4). The compound was labeled in the carboxyl position and had a specific activity of 0.031 mc/mg. The labeled drug was diluted with normal Isoniazid in saline to a concentration of 2.5 mg/ml with a resultant activity of 34 x 10^6 dpm/ml. Adult cats were given an intraperitoneal dose of 10 mg/kg of Isoniazid dissolved in normal saline one hour before being anesthetized with pentobarbital sodium. During anesthesia the chest was opened and perfusion with normal saline begun in order to remove the vascular contents from the capillary bed of the brain. The left ventricle of the heart was cannulated and the descending

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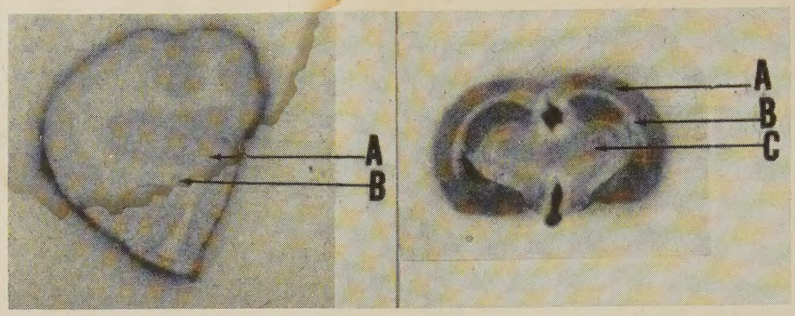

FIG. 1 (left). Autoradiogram of cat brain hemisphere showing density difference between cortex (A) and underlying white matter (B). Peripheral rim of darkest density could represent the very active spinal fluid in subarachnoid space or activity emanating from perpendicular edge of tissue block, or both. Development time, 30 days. Mylar membrane interposed between brain slice and photographic emulsion.

FIG. 2 (right). Autoradiogram of coronal section of rat brain illustrating density difference between cortex (A) and subcortical white matter (B), and showing intermediate density of thalamus (C). Development time, 30 days. No Mylar membrane used. Centrally placed, irregular dark areas represent artifact which is eliminated by the use of Mylar.

aorta was clamped. The right heart was opened to allow return of the perfusate. Two hundred ml of saline was permitted to flow through the system during a period of about 5 minutes. The perfused brains were then carefully removed and placed in a deep freeze to facilitate slicing. Serial coronal sections of approximately 3 mm thickness were made freehand with a 10-inch Valet razor blade, and one hemisphere selected for autoradiography. For C-14 assay purposes, grossly visible anatomical structures were usually selected from the opposite hemisphere, but in some cases it was necessary to obtain additional tissue from the adjacent slice in the same hemisphere. It was found impractical to separate some of the smaller structures (globus pallidus-putamen), and these are represented as single tissues in the accompanying table. Prior to assembly, the tissue slices to be used for autoradiography were placed on a glass slide and chilled to –26°C in a deep freeze, together with black plastic slide boxes (Clay Adams Co., Inc., Cat. No. A1604 B) and foam rubber inserts. The unit was assembled in the dark room by placing Kodak No Screen X-ray film on the floor of the box, covering the free surface of the tissue on the slide with a thin Mylar (du Pont) membrane, and then quickly apposing the tissue and film with the interposed membrane. In our experience the most suitable protective membrane was one quarter mil type A Mylar, which screened out 26% of the beta particles as measured in an open window gas flow Geiger counter. Firm apposition of the tissue slice to the plastic protected photographic emulsion was maintained by placing the foam rubber insert over the inverted glass slide and adherent tissue before closing the slide box. The assembled units thus prepared were sealed with black electrical tape (Minnesota Mining and Mfg. Co. No. 33), and stored at –26°C for 30 days to allow development of the autoradiogram. It is imperative that the dark room operation be carried out rapidly in order to prevent warming of the tissue with resultant softening and distortion due to the slight pressure of the foam rubber. If several autoradiograms are to be prepared at the same time, a pan of dry ice may be used in the dark room to keep the boxes chilled and the tissue slices frozen during the required manipulations. After the 30-day exposure, the film was allowed to come to room temperature before processing. The tissue slices may be fixed in neutral formalin for comparative study.

Results. It may be seen from the illustrations (Fig. 1 and 2) that there is a well defined differential density between various anatomical structures of the brain. It is significant that the relative density of the labeled Isoniazid autoradiograms in the various brain areas is similar to the differential radioactivity of the equivalent anatomical areas as

TABLE I. Activity of C-14 Labeled Isoniazid in Brain Tissues of the Cat. See also Fig. 1. Data by direct plating(6).

Tissue	dpm/mg dry wt
Cerebellar cortex	466
Cerebral "	451
Hippocampus	430
Caudate nucleus	373
Thalamus	380
Cerebral white matter	220
Globus pallidus-putamen	233
Pyramids	175
Brain stem	216
Centrum of brain stem	179
Cerebellar white matter	197
Cerebrospinal fluid	3485*

* dpm/ml.

demonstrated by gross dissection and radioassay (Table I). For example, the cerebral cortex shows an activity of 451 dpm as compared with 220 dpm in the cerebral white matter when calculated on a dpm/mg of dry weight basis. No comparable densitometer measurements of the films were made.

It is to be noted that those areas of localization of C-14 Isoniazid or its metabolites as indicated by the denser areas of the autoradiogram (Fig. 1) correspond to the density of the capillary bed in cortex and white matter of the cat brain, as measured by Hough and Wolff(5). To determine what relationship might exist between drug concentration and vascularity in the central nervous system, time studies with C-14 labeled Isoniazid and other radioactive compounds are in progress. I-131 labeled serum albumin, which is retained within the vascular system, will be used to determine the relative vascularity of various brain areas and the adequacy of the saline perfusion.

Summary. Gross autoradiograms of brain slices containing C-14 labeled Isoniazid, together with supportive data from radioactivity assay, are presented to show the differential localization of Isoniazid in brain tissue, and the technic is described. Future studies to determine the relationship between brain vascularity and brain drug content are indicated.

ADDENDUM: The authors have found that aluminized Mylar gives autograms that are superior to those prepared by using nonmetalized Mylar if more than 30 days exposure time is required.

Aluminized ¼ mil Mylar may be obtained from the Metalized Products Co., Inc., 29 Knight St., Norwalk, Conn.

1. Fitzgerald, Patrick, J., Simmel, Eva, Weinstein, Jerry, and Martin, Cynthia, *Lab. Invest.*, 1953, v2, 181.

2. Levi, Hilde, *Exp. Cell. Res.*, 1954, v7, 44.

3. Manthei, R. W., Roth, L. J., Barclay, W. R., and Ebert, R. H., *Arch int. pharmacodyn.*, 1954, v98, 183.

4. Murray, A., and Langham, W. H., *J. Am. Chem. Soc.*, 1952, v74, 6289.

5. Hough, H. B., and Wolff, H. G., *J. Comp. Neurol.*, 1939, v71, 427.

6. Hogness, J. R., Roth, L. J., Leifer, E., and Langham, W. H., *J. Am. Chem. Soc.*, 1948, v70, 3840.

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Continuous Feed Ligature Carrier. (22259)

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In experiments in our laboratory, where perfusions were made on small animals, ligatures were made around the aorta in 4 places. Since a rather large number of operations were to be performed, a faster method was sought to make these ties. The instrument shown in Fig. 1 was made. In use the needle acts as a probe which once pushed completely under the vessel to be tied supplies the tying thread. The needle is withdrawn and the thread cut with the cutting blade attached to attaching the blade, the needle hub is wrapped with thread, then the blade set in place and wrapped with 21 gauge copper wire. The thread supplies enough springiness so that when the blade becomes dull it can be pushed out and another slid into place on the needle.

The thread is wound into a coil as shown in Fig. 2. A motor drives the tapered glass rod for coiling the thread which is held lightly at the small end with the fingers. Six yards of triple zero surgical silk is wound in a coil 10 cm long while moving the string back and forth. Separate turns of the thread should be about ⅛ inch apart. When the coil is finished, the side thread B is passed under a previous turn and pulled tight. A glass rod about 1 to 1.5 mm diameter is placed against the end of the thread coiler and the coil pushed onto the rod for storage. When the ligature carrier is to be threaded, the free inside end A is pushed through the syringe and used to pull the coil and rod into the syringe. When all is in the syringe, the glass rod is withdrawn and the thread unwinds freely from the inside of the coil. The instrument is ready to use when the threaded needle is attached.

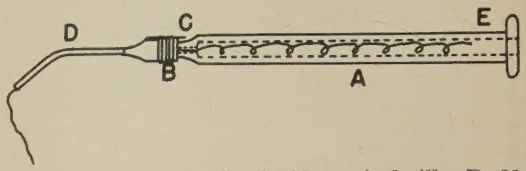

FIG. 1. A, 6 yard coil of 000 surgical silk; B, 21 gauge copper wire; C, steel cutting blade; D, 18 gauge hypodermic needle; E, 1 ml tuberculin syringe.

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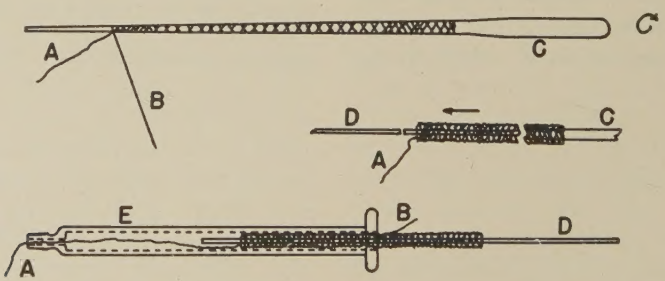

FIG. 2. A, silk thread to inside; B, outside thread; C, thread coiler; D, storage rod; E, 1 ml tuberculin syringe.